

# *A proteomics survey of human articular chondrocytes in osteoarthritis*

Apr. Stijn Lambrecht

December 2007

Promotor:  
Prof. Dieter Deforce  
Prof. Dirk Elewaut

Faculty of Pharmaceutical Sciences  
Laboratory for Pharmaceutical Biotechnology

Thesis submitted in fulfillment of the requirements for the degree of doctor in  
Pharmaceutical Sciences

## Abbreviations:

<b>2-DE,</b>	2-dimensional electrophoresis
<b>ACPA,</b>	anti-citrullinated protein/peptide antibodies
<b>BMP,</b>	bone morphogenetic protein
<b>CAM,</b>	cell associated matrix
<b>CID,</b>	collision-induced dissociation
<b>CRP,</b>	C-reactive protein
<b>DMEM,</b>	Dulbecco's modified Eagle medium
<b>DIGE,</b>	difference gel electrophoresis
<b>ECL,</b>	enhanced chemiluminescence
<b>ECM,</b>	extracellular matrix
<b>ESI,</b>	electrospray ionization
<b>FLS,</b>	fibroblast-like synoviocytes
<b>FPA,</b>	forward-phase protein microarray
<b>HBSS,</b>	Hank's balanced salt solution
<b>HRP,</b>	horseradish peroxidase
<b>IEF,</b>	iso-electric focusing
<b>IF,</b>	immunofluorescence
<b>IGF-1,</b>	insulin-like growth factor 1
<b>IL-1,</b>	interleukin-1
<b>IPG,</b>	immobilized pH gradients
<b>LC,</b>	liquid chromatography
<b>MALDI,</b>	matrix-assisted laser desorption/ionization
<b>MLS,</b>	macrophage-like synoviocytes
<b>MRM,</b>	multiple reaction monitoring
<b>Mw,</b>	molecular weight
<b>MS,</b>	mass spectrometry
<b>NGS,</b>	normal goat serum
<b>OA,</b>	osteoarthritis
<b>PAGE,</b>	poly-acrylamide gel electrophoresis
<b>PBS,</b>	phosphate-buffered saline
<b>pI,</b>	isoelectric point
<b>PTM,</b>	post-translational modification

<b>RA,</b>	rheumatoid arthritis
<b>RNAi,</b>	RNA interference
<b>ROS,</b>	reactive oxygen species
<b>RPA,</b>	reverse-phase protein microarray
<b>RT-PCR,</b>	reverse-transcriptase polymerase chain reaction
<b>SELDI,</b>	surface-enhanced laser desorption/ionization
<b>sHSP,</b>	small heat-shock protein
<b>siRNA,</b>	short interfering RNA
<b>SJIA,</b>	systemic juvenile idiopathic arthritis
<b>SLE,</b>	systemic lupus erythematosus
<b>SpA,</b>	spondylarthropathies
<b>TGF-<math>\beta</math>,</b>	transforming growth factor $\beta$
<b>TMA,</b>	tissue microarray
<b>TNF-<math>\alpha</math>,</b>	tumor necrosis factor $\alpha$
<b>TOF,</b>	time-of-flight



## Table of Contents

### Chapter I, Introduction

Part 1: <i>Proteomics in Rheumatology: the beginning of a fairy tale?</i>	9
Part 2: <i>Osteoarthritis: epidemiology and pathogenesis</i>	27
Part 3: <i>Proteomics: general principles and technical platforms</i>	39

Chapter II, Aim and outline of the study	59
--	----

### Chapter III, Results

Part 1: <i>Differential proteome analysis of normal and osteoarthritic chondrocytes reveals distortion of vimentin network in osteoarthritis</i>	63
Part 2: <i>Regulation of matrix gene expression by the small heat-shock protein, <math>\alpha</math>Bcrystallin, during the development of osteoarthritis</i>	89
Part 3: <i>The proteome of human articular chondrocytes explored by a shotgun proteomics approach</i>	109

Chapter IV, Summary and future perspectives	127
---	-----

Supplementary tables and figures are available on the enclosed CD



# **Chapter I: Introduction**

*Part 1: Proteomics in Rheumatology: the beginning of a fairy tale?*





## **Proteomics in rheumatology: the beginning of a fairy tale?**

Stijn Lambrecht<sup>1</sup>, Kelly Tilleman<sup>1</sup>, Dirk Elewaut<sup>2</sup>, Dieter Deforce<sup>1</sup>

<sup>1</sup>Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium; <sup>2</sup>Laboratory of Molecular Immunology and Inflammation, Department of Rheumatology, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

**Corresponding author:** Prof. Dieter Deforce; Laboratory of Pharmaceutical Biotechnology, Harelbekestraat 72, B-9000 Ghent, Belgium; e-mail: [dieter.deforce@ugent.be](mailto:dieter.deforce@ugent.be); Fax: +32-9-220-6688

## Abstract

One of the major challenges in proteome research is to translate its applications to the setting of human diseases. Proteomics in rheumatology is an area with marked potential including applications ranging from diagnostics, over therapeutic monitoring to discovery of new potential therapeutic targets.

Biomarkers will be essential to discriminate between clinical similar rheumatic diseases, to monitor disease-states or to install the best appropriate therapy. Especially in the field of rheumatology, analysis of specific genes and/or their expression products by pharmacogenetics/-genomics or pharmacoproteomics could be necessary to enable an effective, patient-tailored therapy.

In rheumatology, direct examination of proteins may be of the utmost importance, as it is already known that post-translational modifications, such as citrullination of proteins or peptides, may be involved in certain rheumatic diseases. The discovery and validation of anti-citrullinated protein/peptide antibodies and other auto-antibodies in rheumatic diseases using proteome analysis approaches has been described.

Gel-free methods, SELDI-approaches and classic 2-DE approaches have been deployed on body fluids as well as on target tissues in different rheumatic diseases. Proteomics in rheumatology is on the rise and pilot studies have indicated that the application of proteomics based technologies in rheumatic diseases appears to be an exciting example of translational research.

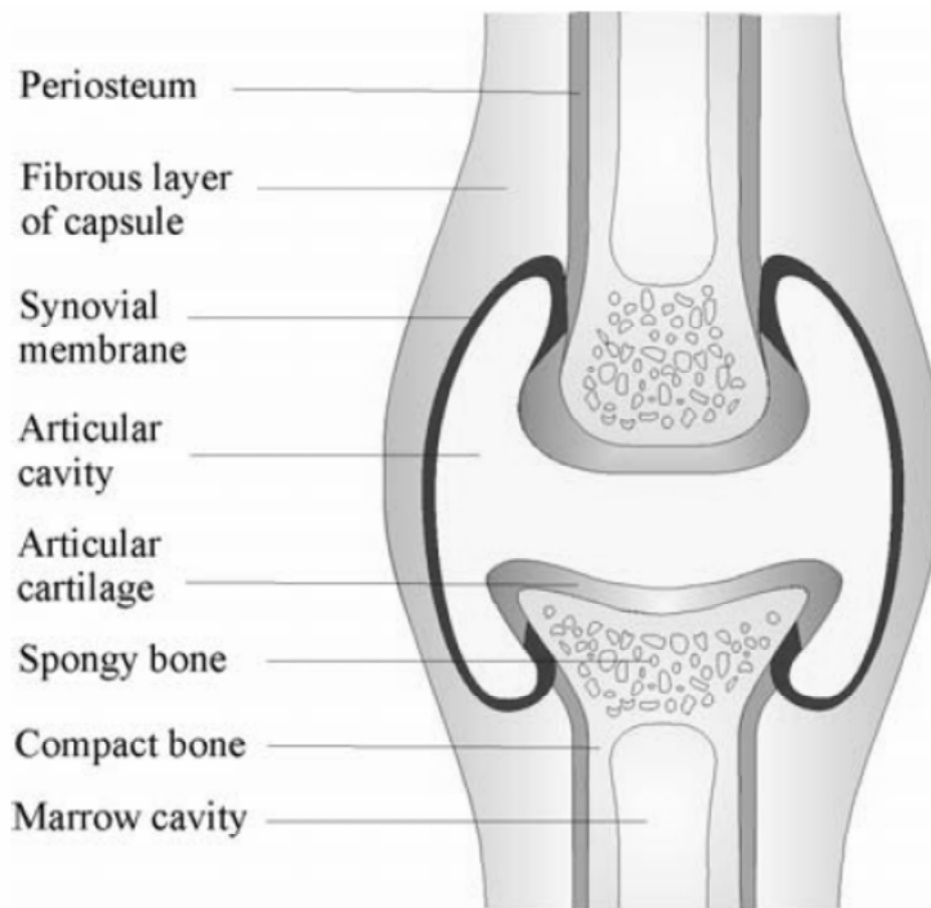
## 1. Introduction

Rheumatology is a discipline of internal medicine which diagnoses and manages a series of related diseases. The primarily targeted organs are the joints. It is therefore obvious that, in addition to serum, the joint tissues are the most frequently investigated by proteomics in rheumatology research, although other organs such as kidneys, lungs and skin may also be affected.

### *1.1 Joint anatomy*

A joint is an organ where two or more bones are “connected” with each other. Joints can be classified based on histological features. Three types of joints are generally discriminated: the fibrous joints, cartilaginous joints and synovial joints. The synovial joints (or diarthroidal joints) are the joints affected by arthritis, the most common symptom in rheumatology.

Synovial joints are surrounded by a capsule that defines the boundary between articular and peri-articular tissues [1] (figure 1). The stability of the joint is provided by the bones, ligaments, joint capsule and the muscles. The articulating bone surfaces have at their ends a thin plate of dense bone; the articular endplate [2]. Adherent to the bony endplate is the hyaline articular cartilage; a specialized smooth connective tissue that is both weight bearing and serves as a gliding surface allowing a lithe movement of the joints. The articular cartilage consists of an extracellular matrix (ECM), which contains and is formed by the only resident cells, the chondrocytes. This tissue is extremely well adapted to bear loads associated with joint use. The articular cartilage is in contact with the joint cavity, which is filled with an aqueous solution of hyaluronan. Together with the vasculature of the subchondral bone, this fluid serves as a nutrient source for the avascular, aneural and alymphatic articular cartilage. In inflammatory conditions, this synovial fluid is actually an ultrafiltrate of plasma with the addition of locally produced hyaluronan, and it is characterized by an accumulation of inflammatory cells such as macrophages, B lymphocytes and T lymphocytes. The amount of synovial fluid in the joint cavity and its content is regulated by the synovial membrane; a vascular connective tissue lining the inner surface of the joint capsule [2]. The synovium consists of two layers: the synovial lining (surface layer) and sublining (the underlining layer) and two distinct synoviocytes can be distinguished: macrophage-like and fibroblast-like synoviocytes [3]. All of the above mentioned structures may be affected during the development of the different forms of arthritis.



**Figure 1:** Schematic representation of a synovial joint, indicating the different bone matrices and the primary targeted articular structures in rheumatic diseases.

## 1.2 *The rheumatic diseases*

As stated above, rheumatology consists of a complex group of related diseases with very heterogeneous clinical appearance and which may occur at any age. Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most frequently occurring and investigated rheumatic diseases.

RA is generally considered as a chronic, inflammatory, autoimmune disorder (for a review see [4] and [5]). It mainly affects the smaller joints in a symmetric way. It is characterized by an inflammation of multiple joints, which ultimately results in the erosion and destruction of joint structures due to the invasion of activated synoviocytes and osteoclast activation. While the primary target of RA is the synovial membrane, advanced RA, however, is accompanied by a progressive destruction of other joint structures such as the articular cartilage and subchondral and periarticular bone [6]. Besides the joint involvement, extra-articular features including vasculitis and subcutaneous nodules may also occur.

Besides RA, a group of other inflammatory joint diseases can be distinguished; the spondylarthropathies (SpA). Clinical symptoms of SpA comprise of inflammatory back pain, asymmetrical oligoarthritis and inflammation of other organs such as the gut, the skin or the eye. Five different subgroups can be discriminated: ankylosing spondylitis, psoriatic spondyloarthritis, reactive spondyloarthritis, spondyloarthritis associated with inflammatory bowel disease and undifferentiated spondyloarthritis [7]. Hallmarks of these types of chronic arthritis are that the destructive properties are simultaneously combined with the formation of new bone. Some groups have therefore termed these types of disorders as an example of destructive and remodelling arthritis.

Juvenile idiopathic arthritis encompasses all forms of arthritis that begin before the age of 16 years, persist for more than 6 weeks, and are of unknown cause. It is a general term that describes a clinically heterogeneous group of arthritides (reviewed in [8]). Interestingly, symptoms of these different forms of arthritis are not limited to the joints, but may include systemic symptoms such as fever, lymphadenopathy and hepatosplenomegaly.

Two rheumatic diseases which do not primarily target the joints are systemic lupus erythematosus (SLE) and Sjögren's syndrome. SLE is a systemic auto-immune disease of unknown aetiology. Symptoms are rather heterogeneous and the targeted organs include the skin, kidneys, muscles, lungs and the heart. Psychological symptoms such as anxiety, depression and fatigue are commonly observed. Sjögren's syndrome is also a systemic auto-immune disease, which primarily targets the exocrine glands resulting in dryness of the eyes and mouth. However, as in SLE internal organs may also be affected by this disease. Furthermore, other systemic diseases such as systemic sclerosis are also followed by rheumatologists.

In contrast to inflammatory arthritides, OA is not considered a classical inflammatory arthropathy, due to the absence of inflammatory cells in the synovial fluid and the lack of systemic signs of inflammation. However, some studies suggested that some signs of synovial inflammation may occur during the course of the disease [9]. It is a slowly progressive disease characterized by a degeneration of the articular cartilage, which mostly affects the large weight-bearing joints (for an extended review see [10]). The cartilage degeneration is the ultimate result of an imbalance between anabolic and catabolic processes in the chondrocyte. Cartilage deterioration combined with other structural changes (e.g. formation of new bone at the joint margins) leads to malalignment of the joint, which ultimately results in joint failure.

### ***1.3 The need for translational research in rheumatology***

Proteome studies in rheumatology mainly focus on two different domains: biomarker discovery and the search for new key players in disease pathogenesis. A biomarker has been defined by the Biomarkers Definition Working Group as: “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention” [11]. This definition implies that biomarkers may not only be used to stratify patients or to predict outcome, but also to predict responders and non-responders to a given therapy.

Probably, the area with greatest potential in rheumatology research is biomarker discovery. It is of utmost importance for a rheumatologist to be able to discriminate and to recognize different disease subtypes. Nowadays, diagnosis is largely based on clinical observations combined with serological analyses and synovial histological features. However, clinical symptoms may develop initially rather atypical and the course of the disease may be very unpredictable. Today, the major challenge remains to predict disease development or evolution, even before the presentation of major clinical symptoms. Particularly in the case of RA, it is known that in the initial phase of the disease, the rate of joint destruction is very high [12]. Synovial activation may occur before the development of clinical symptoms or even persist despite remission of clinical signs, reflecting the need for early-disease markers. In the case of ankylosing spondylitis, there is an average interval from 8-11 years, between the onset of symptoms and the time of diagnosis [13]. The knowledge of early molecular markers could help the clinician to shorten this interval and to implement the appropriate therapy. Good biomarkers may not only serve as early diagnostic tools, but also as predictors of disease progression and therapeutic response. For example, in RA there are very few clear-cut differences between early and late stages of RA and even on the histological level, RA cannot invariably be distinguished from other types of synovitis [3].

A major criterion for a good biomarker is its presence in an easily accessible body fluid. In joint pathologies, synovial fluid serves as an additional body fluid for biomarker-screening. In inflammatory conditions, the synovial fluid is actually an ultrafiltrate of plasma with the addition of locally produced hyaluronan, and it is characterized by an accumulation of inflammatory cells such as macrophages, B lymphocytes and T lymphocytes. As synovial activation is one of the primary manifestations in inflammatory pathologies, such as RA, the synovial fluid is very commonly screened in biomarker discovery studies. As described in the introduction, most rheumatic diseases are characterized as auto-immune diseases. This opens the perspective to use auto-antibodies as potential biomarkers, as will be discussed later.

A second area of translational research in rheumatology is the search for key players in disease pathogenesis. The knowledge of differentially abundant proteins in a target tissue may provide new insights in the pathogenesis, which may open avenues for the rational development of new targeted therapies. For example, in OA no sufficiently satisfactory disease-modifying drugs are available and therefore, drug therapy is mainly limited to the control of symptoms [14]. Thus, the knowledge of the underlying disease processes may be of invaluable significance. In inflammatory pathologies, underlying disease mechanisms can be elucidated by analyzing the synovial membrane which is believed to be one of the initial sites where the disease occurs and progresses. Alternatively, exposing cultured synoviocytes to various inflammatory conditions can provide insight in the inflammatory pathways triggered in the joint. In OA, generally considered as a non-inflammatory disease, the chondrocytes play a pivotal role in the slowly progressive degeneration of the articular cartilage [15]. As a consequence, isolated chondrocytes and the articular cartilage itself are very attractive subjects to study OA pathogenesis.

The results of studying target tissues or body fluids by the use of proteomics techniques are intertwined: analysis of serum or synovial fluid may lead to the discovery of new rheumatic biomarkers but can also provide new insights into the pathogenesis and the knowledge of the disease. In contrast, studying target tissues and the subsequent discovery of disease pathways, may lead to rational biomarker discovery.

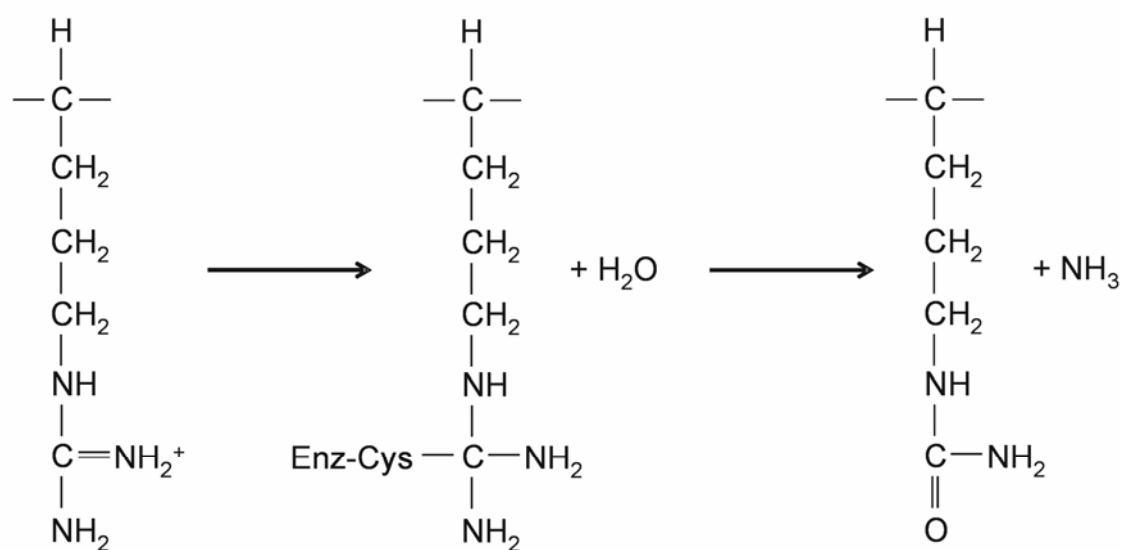
## 2. What did proteomics learn us about rheumatology?

### 2.1 *Serological markers and auto-antigen discovery*

The majority of rheumatic diseases are known as auto-immune diseases. A lot of efforts have been made to characterize auto-antibodies and the antigens they are directed against. Besides a diagnostic value, their identification may provide new insights into the pathogenesis of rheumatic diseases and auto-immune diseases in general.

The most common serological marker for RA is the presence of rheumatoid factor. This factor is an immunoglobulin (Ig) M antibody response directed against the Fc portion of IgG, leading to the formation of complexes [16]. However, this antibody is not very specific for RA (specificity: 74%, sensitivity: 75% [17]) and can be found in many other inflammatory diseases and in healthy elderly individuals [18]. In recent years, a variety of additional auto-antibodies have been described in patients with RA (reviewed in [19]). Antibodies against

citrulline containing proteins (ACPA) have shown to be highly specific for RA (specificity: 96-98 % [20, 21]), without compromising their sensitivity. They can be observed in the serum of patients before clinical onset [22, 23]. During citrullination, arginine residues are post-translationally, enzymatically converted into citrulline residues (figure 2). A detailed reaction mechanism for this enzymatic conversion was proposed by Arita *et al* [24]. The reaction is catalyzed by the enzyme peptidylarginine deiminase (PAD). The arginine residue is targeted via a cysteine residue of the enzyme, yielding a tetrahedral adduct. Nucleophilic attack by water results in the formation of ammonia and the citrulline residue, which differs 1 Da in mass from the original arginine (figure 2).



**Figure 2:** Illustration of the reaction mechanism, describing the enzymatic conversion of an arginine residue into citrulline. The reaction is catalyzed by the enzyme peptidylarginine deiminase (PAD). This enzyme targets the arginine residue via a cysteine residue, yielding a tetrahedral adduct. A nucleophilic attack of water results in the formation of the keto-group and release of the cysteine residue. The resulting citrulline residue differs 1 Da in mass from the original arginine.

Besides the autoantibody response, there is some indirect evidence suggesting a more important role for protein citrullination in the pathogenesis of RA since ACPA can be observed in the sera of patients before clinical onset [22, 23]. Anti-CCP (anti-cyclic citrullinated peptides) producing plasma cells have been shown in the inflamed synovial RA tissue [25] suggesting the presence of citrullinated proteins in the synovial membrane causing an antigen driven maturation of ACPA-specific B cells at the site of inflammation. Indeed, the presence of citrullinated proteins, like fibrinogen  $\alpha$  and fibrinogen  $\beta$ , have been shown in the RA synovium [26]. However, these forms of fibrin are not specific for RA but are also



observed in non-RA synovium [27]. The presence of ACPA is also strongly associated with a specific Major Histocompatibility Complex background: HLA-DR shared epitope positive RA patients have higher ACPA levels [28].

However, it is still largely unknown, what the exact role is of protein citrullination in arthritis, as PAD expression and citrullination of proteins are found in other tissue and pathologies [29, 30]. This process of arginine transformation could be a more universal characteristic of inflammation, although very little is known about the function of protein citrullination in general.

Proteomics techniques are very well suited to discover post-translational modification of antigens. In the recent years 2-DE has repeatedly been used to elucidate antibody responses especially to modified proteins and it still holds great promise in the discovery of new auto-antigens which is a hot topic in the auto-immune disease research. In general, tissue proteins are separated by 2-DE followed by western blotting and incubation with sera of patients.

For the discovery of new citrullinated synovial auto-antigens, such an approach was applied by Matsuo *et al* [31]. Thirteen citrullinated auto-antigenic protein spots were identified in a RA synovial tissue sample. The usefulness of such 2-DE approach was also demonstrated by our own group [32]: protein patterns of some immunoreactive spots appeared as “trains” on the 2-D gel. Subsequently, these protein modifications were identified as citrullinated subunits of fibrinogen. Other 2-DE based approaches, suggested that auto-antibodies against aldolase A [33] and  $\alpha$ -enolase [34] are also associated with RA. The authors of the latter study suggested that RA sera showed autoreactivity predominantly to a posttranslationally modified or appropriately folded epitope of  $\alpha$ -enolase. Kinloch *et al.* confirmed  $\alpha$ -enolase as a potential auto-antigen in RA, more specifically the citrullinated form of  $\alpha$ -enolase [35]. Undoubtedly, post-translational modifications can induce a break in tolerance and can initiate the production of antibodies. It is not unlikely that in the near future, besides citrullination, other antigen modifications will be identified by proteomics techniques which are associated with the development of auto-immune diseases. 2-DE approaches should still be considered as a very powerful tool in the discovery of new auto-antigens as they can precisely indicate against which protein spot the specific auto-antibodies in the sera of patients react.

An alternative approach, using antigen microarrays, demonstrated that citrullinated epitopes were preferentially targeted by auto-antibodies present in serum of patients with features predictive of severe arthritis. In contrast, autoreactivity against a set of native auto-antigens

could discriminate a subpopulation of patients with early RA with features predictive of mild arthritis [36]. Another microarray analysis by the same group showed clear association between anti-citrulline reactivity and elevated blood levels of pro-inflammatory cytokines in patients with early RA [37]. Feng *et al.* used arrays loaded with 15 known auto-antigens in rheumatic diseases and incubated them with serum samples from patients with different autoimmune rheumatic diseases. Given the reported correlation of specificity and sensitivity with previously reported data, the authors present such arrays as a potential high-throughput diagnostic tool, which allows parallel screening of different auto-antigens [38].

Whereas autoimmune processes are well described in RA, OA is generally considered as a non-inflammatory, degenerative disease. However, it has been suggested that autoimmune processes directed against cartilage components might play a role in the cartilage destruction associated with OA [39]. Fibulin-4, a cartilage matrix protein, has been identified as a potential auto-antigen in OA and might play a role in OA pathogenesis [40]. The same group described triosephosphate isomerase as an auto-antigen in OA as revealed by a proteomics study [41].

In addition to RA and OA, proteomics-based auto-antibodies discovery studies have also been deployed to investigate lupus disease activity. Glomerular proteome arrays, containing about 30 antigens documented to be present in the glomerular milieu, unravelled several IgG and IgM auto-antibody clusters. Two of the IgG antibody clusters were found to correlate well with disease severity, whereas one IgM cluster was associated with reduced disease severity [42]. One of the major disadvantages of such antigen-arrays, is the difficulty to unravel novel auto-antigens, as they contain only a limited number of presented antigens.

Especially in the field of the inflammatory diseases, auto-antibody discovery is of the utmost importance, not only as a diagnostic marker, but also to provide new insights in the pathogenesis of different related diseases. Pilot proteomics-based studies have confirmed known phenomena and provided novel potential interesting targets. Probably, the greatest potential of such studies lies in the ability to detect different isoforms of a single protein, as it has become clear that post-translational modifications have a great impact on the development of auto-antibodies.

## **2.2    *The search for biomarkers in serum/plasma or synovial fluid***

Several groups have analysed synovial fluid, serum or plasma samples using 2-DE in order to find biomarkers which can distinguish RA from other joint diseases [43, 44]. Based on a

differential proteome study, the authors of the latter study validated the applicability of the S100A8/S100A9 heterocomplex as a diagnostic marker for RA using ELISA. The S100A9 protein was also identified in the former study as differentially expressed in RA synovial fluid compared to OA. In addition, the authors reported the selective presence of serum amyloid A in RA synovial fluid and plasma. A single biomarker, which distinguishes RA from all other diseases, is probably unrealistic. It is more likely that a combination of proteins will be more conclusive. Dotzlaw *et al* [45] used a subset of 29 differentially expressed proteins in peripheral blood mononuclear cells for a hierarchical cluster analysis, enabling total segregation of patients and controls.

As stated before, a set of biomarkers, disclosed by proteome analysis, is not only useful for patient stratification but also to monitor therapy response. Smith *et al* [46] were the first to demonstrate this principle in the field of rheumatology: levels of acute-phase proteins in synovial fluid, quantified after 2-DE separation, correlated well with clinical conditions and other parameters of disease progression after anti-CD4 administration.

Synovial fluids of OA patients have also been screened by 2-DE, however no comparison was made with other diseases [47]. An exploratory study investigated synovial proteomes of children with single and recurrent joint inflammation and compared these patterns with corresponding plasma samples, in an attempt to identify proteins which were over-expressed in the joints or in recurrent inflammation [48].

In recent years, a lot of effort has been made to omit the labour-intensive 2-DE gels. Also in the field of rheumatology, gel-free methods have been applied in the search for suitable biomarkers. A SELDI TOF-MS analysis compared synovial fluids of RA and OA patients, revealing several biomarker candidates for RA [49]. After further purification, identification and validation experiments the authors proposed the S100A8 protein as a candidate biomarker. These S100 proteins have been associated with several other inflammatory diseases and it is now generally accepted that these proteins may have proinflammatory functions in several autoinflammatory disorders [50]. Not surprisingly, these S100 proteins have been identified in several rheumatic biomarker screening studies as differentially expressed proteins. Altogether, these proteins should be considered as markers of the inflammatory disease state, rather than specific RA-biomarkers. One of the major disadvantages of a SELDI platform is that it does not allow direct identification of the potential interesting spectrum peaks. In experimental settings, a cluster of unidentified peaks may be used to discriminate RA-patients from controls [51] or active versus inactive lupus disease states [52]. Miyamae *et al* [53] performed a SELDI analysis on serum samples of

systemic juvenile idiopathic arthritis (SJIA) patients, demonstrating the potential of such an approach to discriminate between different disease states based on a set of peaks. One of these peaks was subsequently identified as serum amyloid A, a known acute-phase reactant in SJIA. In addition, the authors presented a set of unidentified peaks predictive for therapy response. While these studies are very promising in experimental settings, it has to be kept in mind that it is very unlikely that diagnostic tests will be used in clinical practice without the knowledge of biomarker identity. Therefore, these studies should be regarded as proof-of-principle rather than an endpoint.

SELDI approaches have also been used in the search for biomarkers in Sjögren's syndrome. SELDI combined with a 2-D differential gel electrophoresis (2-D DIGE) was applied to compare saliva samples from Sjögren's Syndrome-patients with healthy subjects. This revealed a set of previously described and novel potential biomarkers, of which lactoferrin and  $\beta$ 2-microglobulin have been validated by ELISA [54]. Tear proteomic patterns in Sjögren's Syndrome have been investigated by SELDI TOF-MS, yielding a set of protein peaks with potential predictive value for Sjögren's Syndrome diagnosis [55].

NanoLC-MS/MS methods were introduced in rheumatology biomarker research by Kuhn *et al* [56]. They demonstrated the ability of a nanoLC-MS/MS platform, based on multiple-reaction monitoring (MRM), to quantitate C-reactive protein (CRP), a known diagnostic marker for RA, in serum samples. In a later study [57], the same group combined this MRM approach to validate candidate markers, selected by a differential 2-D nanoLC-MS/MS screening analysis. Among these candidates were proteins known to be associated with inflammatory processes, such as S100 proteins and CRP, demonstrating the potential as a discovery tool.

### ***2.3 The search for mechanisms involved in pathogenesis: proteome analyses of target tissues***

In inflammatory pathologies, disease modifying drug therapies have been developed in the recent years, although the disease mechanisms are not yet fully understood. On the other hand, drug treatment for OA, the most common form of joint diseases, is limited to symptom control management without convincing effects on disease progression [58]. Differential proteome analyses comparing different disease states or diseased versus healthy samples might help us to provide new insights in the pathogenesis of rheumatic diseases. In addition, well defined animal models (e.g. rats with surgically induced OA by anterior cruciate ligament transaction) may also assist in the discovery of novel disease pathways. Such approach was recently demonstrated at the RNA level to identify genes involved in OA

pathogenesis [59]. Moreover, given the continuous growth in commercially available knock-out animals, such models may serve as an excellent tool to validate proteome analysis data. Well validated proteome generated data, may ultimately help to unravel disease pathways and assist in drug development. Given the heterogeneity in progression or therapeutic response, the knowledge of detailed rheumatic disease mechanisms undoubtedly will assist in novel therapy developments.

### **2.3.1 Synovial tissue**

Synovial tissue is characterized by the presence of two different cell types: fibroblast-like (FLS) and macrophage-like synoviocytes (MLS) [60]. The synovium is the primarily targeted tissue during inflammatory joint pathologies. In conditions such as RA or SpA the physiological synovium alters into an inflammatory activated synovium [3]. The proteome of cultured FLS derived from RA-synovium was characterized using a 2-DE approach followed by mass spectrometry [61]. However, the authors did not compare the protein expression pattern between different diseases. Our own group compared the expression levels by 2-DE of soluble proteins extracted from RA, SpA and OA synovia [62]. Based on a hierarchical cluster analysis, it was concluded that RA and SpA patients showed a distinguished expression pattern, even though both are inflammatory pathologies. Not surprisingly, the previously described inflammation marker S100A8, together with other proteins, showed a differential expression between the inflammatory and OA synovia. Using an alternative approach in which transcriptomics (using gene microarrays) and proteomics were combined (using multi-Western PowerBlot), Lorenz *et al* described 58 differentially expressed proteins between RA and OA synovial samples [63]. Among these, the protease cathepsin D showed marked down-regulation in RA when compared to OA tissues.

### **2.3.2 Cartilage**

Cartilage is a very attractive tissue to molecular biologists, as it contains only a single cell type. At the same time, it forms a major challenge for proteome analysts. This single cell type comprises only 1% of the total volume [64], while the main components of the cartilage tissue are highly abundant extracellular matrix (ECM) proteins (proteoglycans and collagens). Recently, protein profiles of human articular cartilage were determined using a combination of 1-DE prefractionation and LC-MS/MS [65] or using a 2-DE approach preceded by an extensive sample preparation procedure [66]. A proteome map of cultured human chondrocytes was presented by Ruiz-Romero *et al.* [67], followed by the study of the

subcellular proteome of cultured chondrocyte mitochondria [68]. All these studies revealed interesting information, and provided new insights in the protein content of the ECM and chondrocytes. However, no comparison was made between healthy and diseased samples. Such differential proteome analysis of proteins secreted by cultured chondrocytes revealed novel potential regulatory molecules for articular cartilage, such as inhibin betaA [69]. Our own group performed a 2-DE based differential proteome analysis to compare protein expression patterns of cultured normal chondrocytes versus OA chondrocytes. A marked cleavage of vimentin in OA samples was detected by the 2-DE analysis. Subsequent validation experiments clearly pointed to distortion of the vimentin organisation associated with OA. In addition, novel players were identified in processes known to be involved in OA pathogenesis, such as oxidative stress, apoptosis and metabolic activation ([70], *in press*). Undoubtedly, the results generated by such differential analyses will further contribute to our knowledge of processes activated in OA-chondrocytes.

### 3. Future perspectives

Large scale protein analyses are being conducted on a variety of biological material affected in degenerative and inflammatory joint pathologies. This translational research field aims to identify new biomarkers (for patient stratification, prediction of therapeutic response, disease progression, ...) and to gain insight in these complex multifactor diseases, which may assist in rational drug development.

The above discussed studies, resulted in the identification of several potentially interesting proteins. However from the therapeutic point of view, integrated approaches, combining proteome results with disciplines as RNA-interference and interactomics are needed to assess the biological value of the presented candidates. Only in this way, the aim of translating proteomics from bench to bedside will be achieved at the level of rational drug development.

From the biomarker perspective, it is absolutely crucial to set out definite goals for each study conducted and to use clinically well defined samples. A major issue herein is the definition of the methods used for serum preparation, as it is generally known that the peptide signature of serum samples is influenced by the method used for serum preparation [71]. Another issue is the isolation of synovial fluid: if the isolation is not well performed, blood contamination may become an issue skewing the results. The definition of standard methods would greatly enhance the transparency of the obtained data.

For the future, we feel that there are two important issues that should be set out in this field:

First of all, for serological markers and auto-antigen discovery it is of the utmost importance to be able to identify the *in vivo* epitope against which the antibodies react. For example, in the case of citrullination, it is still unknown what the *in vivo* citrullinated status of the proteins in the joint is. Although it is possible to visualize citrulline containing proteins by specific detection methods [72], the specific residues which are citrullinated residues in these proteins remain to be localised. Proteome analysis combined with mass spectrometry could elucidate the *in vivo* citrullinome in the joint of several rheumatic diseases and bring us one step closer to understanding how these post-translational modifications are implied in the pathology.

Secondly, biomarker research in the field of rheumatology is often conducted on samples derived from OA and RA patients. The possibility to discriminate between a non-inflammatory and inflammatory pathology is important but can be achieved by other means, primarily on radiographic and histological features. The true goal of translational research in a rheumatic setting should lie, in our opinion, in the ability to find biomarkers to distinguish interrelated inflammatory joint pathologies which are still very difficult to discriminate.

## Acknowledgements

S. Lambrecht is supported by a grant of the Fund of Scientific Research-Flanders (Belgium). Dr. K. Tilleman is a post-doctoral fellow with the Fund of Scientific Research-Flanders (Belgium). We acknowledge Dr. K. Coppieters for the joint illustration.





# **Chapter I: Introduction**

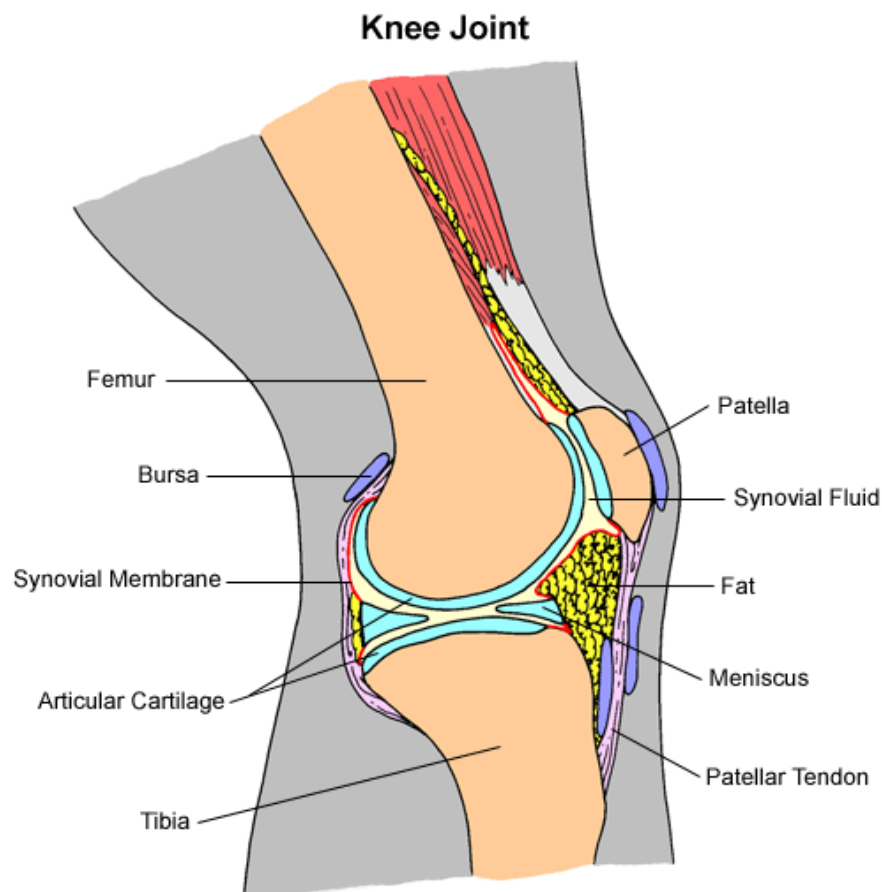
*Part 2: Osteoarthritis: epidemiology and pathogenesis*

## Abstract

The articular cartilage is a highly specialized tissue, extremely well adapted to take up and distribute load associated with joint use. Osteoarthritis is the most common disease that affects this tissue. One of the major hallmarks of this disorder is the slowly progressive degeneration of the cartilage matrix, ultimately leading to joint failure. A substantial amount of knowledge is available about processes associated with osteoarthritis. Unfortunately, at this time this information did not result in the availability of sufficiently satisfactory disease-modifying drugs.

## 1. Articular cartilage, a highly specialized tissue

Osteoarthritis (OA) is primarily a disease of the articular cartilage. Articular cartilage is a specialized tissue that forms the smooth, gliding surface of diarthroidal joints. Diarthroidal joints (also called synovial joints) permit a variety of movements and have a joint cavity between the articulating bones (figure 3). The articular cartilage is bathed in the synovial fluid that serves, together with the vasculature of the subchondral bone, as a nutrient source for the chondrocytes. Cartilage consists of an aneural, avascular and alymphatic extracellular matrix (ECM), which is synthesized by the sparsely distributed cells, the chondrocytes [73]. In human adults, these chondrocytes are considered as terminally differentiated cells which are relatively metabolic inactive [74].

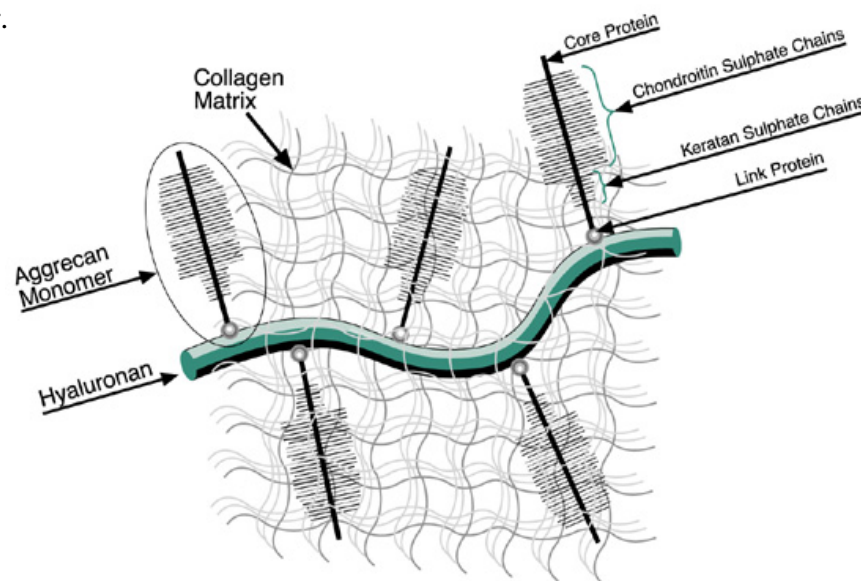


**Figure 3:** Simplified, schematic representation of a knee joint. (Obtained from [www.healthcare.utah.edu](http://www.healthcare.utah.edu))

The major constituents of the extracellular cartilage matrix are water, proteoglycans and collagens. Proteoglycans are formed by a protein core with glycosaminoglycan side chains, mostly keratan sulphate and chondroitin sulphate. Aggrecan is the most abundant proteoglycan. Large aggrecan monomers (200-300 kDa core protein) interact with hyaluronic

acid resulting in proteoglycan aggregates. They constitute the nonfibrillar component of the cartilage matrix (Figure 4). Together with aggrecan, collagen type II is the most abundant matrix molecule. Collagen type II forms, together with less abundant collagens, the fibrillar matrix, providing compressive stiffness [75]. Next to these constituents, a whole scale of less abundant matrix components is present, such as fibronectin, tenascin, thrombospondin,... These components give rise to a tissue extremely well adapted to take up and distribute load with minimal deformation. These complex organisations are assembled outside the chondrocyte in the extracellular environment. The chondrocytes are dispersed throughout this matrix and tightly control this complex network of biomolecules.

The ECM in adult cartilage is organized in several compartments [76]. The pericellular matrix is located immediately adjacent to the chondrocytes. More distal from the chondrocytes is the territorial matrix, which composes, together with the pericellular matrix, the cell-associated matrix (CAM). The metabolically most inert zone of the articular cartilage is the interterritorial matrix. All of these individual zones differ in composition and organisation of the matrix compounds. As stated before, chondrocytes are the only resident cells in the cartilage and are considered to be terminally differentiated cells that maintain the cartilage matrix, at least under normal conditions of low turnover [15]. The turnover rate of collagen is very slow in contrast to the proteoglycans, which are continually resynthesized. The homeostasis of the extracellular matrix (ECM), mainly depends on the response of the chondrocyte to autocrine and paracrine anabolic and catabolic processes, which will be discussed later.



**Figure 4:** Schematic presentation of the major ECM compounds of articular cartilage. Obtained from [www.peprotech.com](http://www.peprotech.com)

## 2. Osteoarthritis: Clinical perspective and current treatment options

### 2.1 *Definition of OA*

In 1995 the following consensus definition was proposed to define osteoarthritis: “Osteoarthritic diseases are a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix, and subchondral bone. Although they may be initiated by multiple factors, including genetic, developmental, metabolic, and traumatic, OA diseases involve all of the tissues of the diarthrodial joint. Ultimately, OA diseases are manifested by morphologic, biochemical, molecular, and biomechanical changes of both cells and matrix which lead to a softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes and subchondral cysts. When clinically evident, OA diseases are characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation without systemic effects.” [77].

### 2.2 *Epidemiology and risk factors of OA*

Osteoarthritis (OA) is a major health problem throughout the western world. For example, the prevalence of knee-OA has been estimated to be between 28-34 % in Spain [78]. Associated herewith, is a major cost. Because of longer working careers and the relative high frequency of osteoarthritis in middle-aged persons, OA results in a considerable loss of time at work and early retirement [79]. For instance, the estimated direct cost of OA in France was 1.64 billion euros in 2001 [80].

Prevalence and severity of OA parallel age in most individuals. It is assumed that almost everyone shows radiographic changes of knee-OA after the age of 75. However, most individuals are non-symptomatic. Next to age, several other risk factors have been associated with the development of OA such as sex, ethnic characteristics, bone density, genetics, estrogen replacement therapy, obesity, muscle weakness,... [58]. For example, before 50 years of age, the prevalence of OA in most joints is higher in man than in women. The opposite is true after the age of 50. However, hip OA is more frequent in men [79]. Another example is obesity, which is probably one of the most important risk factors. Obese subjects are at very high risk to develop knee-OA [81]. Given the rise in average body weight in recent decades [82], it may be expected that obesity related knee OA will increase in number as in severity in the near future. Not surprisingly, several studies showed that weight reduction may result in a relief of symptoms associated with OA (reviewed in [83]).

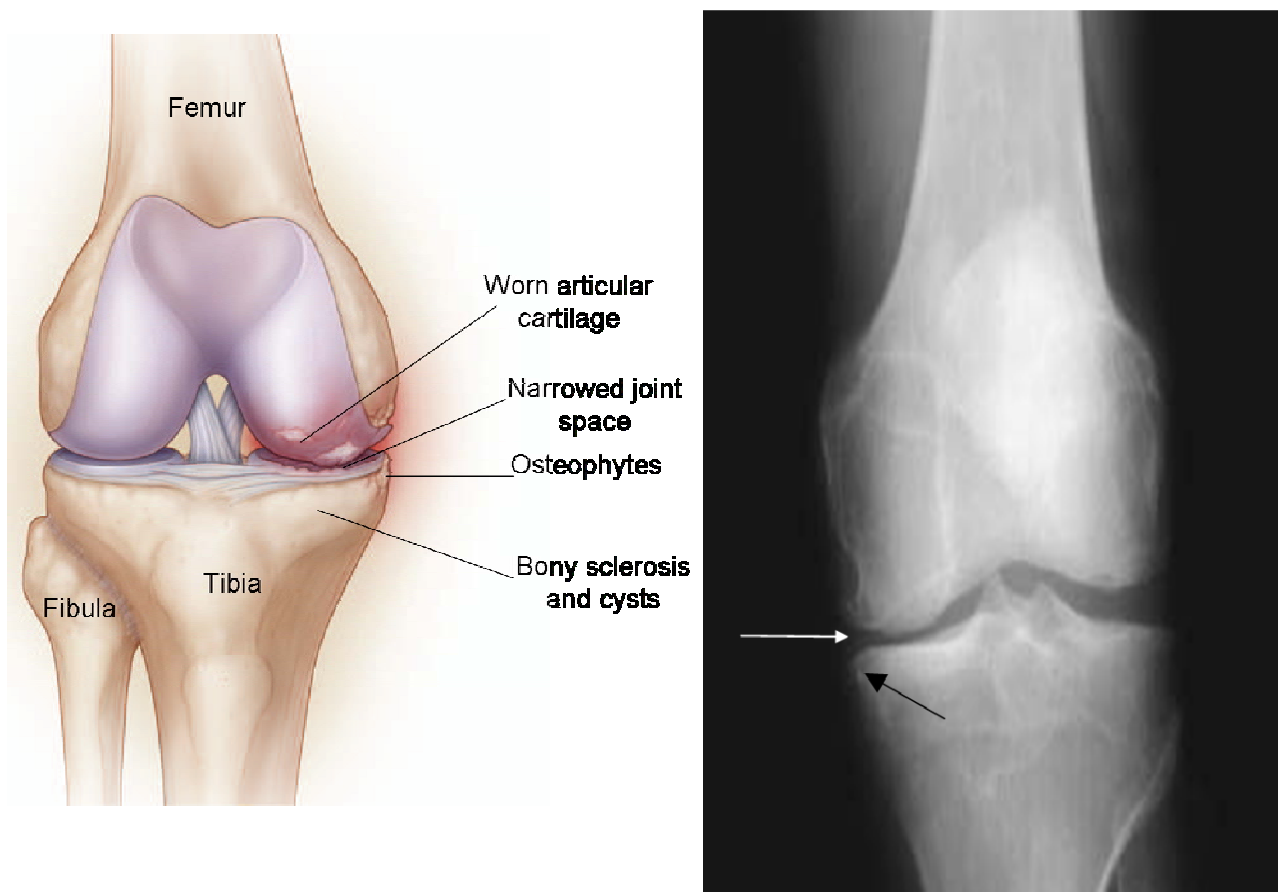
In recent years, several groups performed gene polymorphism studies to unravel genes associated with the susceptibility to OA. Efforts have been exercised to associate gene polymorphisms in ECM structural protein genes with OA-susceptibility. However, most reports provide little or no evidence for such association. In contrast, polymorphism in genes encoding proteins that have a regulatory function showed promising results (reviewed in [84]). The group of Ikegawa performed several population studies in Asian people and identified associations of several gene polymorphisms (GDF5 [85], a growth factor of the BMP-2 family; CALM1 [86], a gene involved in signal transduction; and ASPN [87], which has been shown to modulate the expression of collagen type II and aggrecan) with the susceptibility to OA. In addition, a large scale multi-center study indicated a role for genes encoding the cartilage matrix proteins COMP and COL2A1, an antagonist of Wnt signaling (FRZB) and the ASPN gene. Furthermore, this study emphasized the variation between populations and sexes [88]. This variation was confirmed in an additional study, highlighting the heterogeneous nature of OA genetic susceptibility [89]. Finally, these and several other groups published additional genes which might be associated with OA, among these are the IL-4R [90] and IL-17 [91] genes.

Whereas OA has long been considered as being a result of trauma or age, it is now generally accepted that the etiology of OA is multiple and includes various mechanical, biochemical and genetic factors [74].

### **2.3    *Clinical features***

From a clinical perspective, this pathology is typically associated with pain that occurs with joint use. The most prominent sources of pain are the bone, synovial inflammation and a stretched joint capsule filled with fluid. After all, the pathology of osteoarthritis affects all structures within a joint: The characteristic structural changes associated with the development of OA include a progressive loss of articular cartilage, increased subchondral plate thickness, formation of new bone at the joint margins (osteophytes) and the development of subchondral cysts [92] (figure 5a). Furthermore, in some patients synovitis and lesions in the bone marrow may develop. Such macroscopic structural changes are usually observed by radiography and may be used as a confirmation in the diagnosis of OA (figure 5b). In the recent decade, another imaging technique, magnetic resonance imaging (MRI), has evolved to a very promising diagnostic tool for diagnosis as well as follow-up of disease progression [93]. Localized areas of loss of cartilage can increase focal stress across the joint, leading to further cartilage loss, creating a vicious cycle of joint damage that ultimately can lead to joint

failure [94]. OA is generally considered to be a disease of the articular cartilage. However, a recent editorial suggests that abnormalities in the surrounding environment (ligaments, bone, nerves, meniscus and muscles) are at least as important targets for the development of disease-modifying therapy for OA as the cartilage [95]. In conclusion, it may be suggested that the degenerative process in the articular cartilage may be initiated in a variety of ways and by a variety of joint constituents, as any constituent of the joint may affect the other.



**Figure 5:** Next to articular cartilage degeneration, other macroscopic changes such as the development of osteophytes may be observed. Figure 5a (left panel): Schematic representation of an OA-affected knee at the medial side (adapted from [94]). Figure 5b (right panel): Radiograph showing cartilage degeneration of the medial side of the knee. White arrow indicates narrowing of the medial joint space; black arrow indicates the formation of osteophytes. (obtained from [94]).

## 2.4 Current treatment options for OA

Today, there is no known cure for OA of the knee or hip. The major goal of current treatments is to control pain and to improve quality of life. The analgesic acetaminophen is generally considered as an initial treatment option, based on its overall cost, efficacy and toxicity [96].

Compared to NSAIDs, acetaminophen is as efficient in pain relief in patients with mild to moderate pain [97, 98]. Even though, acetaminophen often fails to control pain beyond that of the mild or moderate degree. Therefore, in patients with moderate-to-severe pain, and in whom signs of joint inflammation are present, a NSAID or joint aspiration combined with an intra-articular injection of glucocorticoids is a valuable alternative as an initial approach [99]. Recently, a group of 'nutraceuticals' (mostly glucosamine or chondroitine sulphate) have been proposed as alternative therapies for OA. The information concerning the efficacy of these products is contradictory and greatly depends on the study design. The use of glucosamine as a therapy for OA has been reviewed [100] and some data suggest that these nutraceuticals may improve the symptoms of OA. However, due to sparse data, further studies are absolutely warranted [101]. A recent multi-center, double-blind, placebo- and celecoxib-controlled study (the GAIT-study) showed that glucosamine and chondroitin sulphate alone or in combination did not reduce knee pain effectively in the overall group of patients. However, exploratory analyses suggest that the combination of both may be effective in a subgroup of patients with moderate-to-severe knee pain [102]. ACR-guidelines suggest that all of these pharmacological treatments should be considered as additives to nonpharmacological measurements such as physical therapy, weight loss, patient education,... [99]. Next to the ACR-guidelines, the EULAR proposed its own guidelines for OA-management depending on the affected joint: hand [103], hip [104] or knee (see table 1) [105]. If patients with severe symptomatic OA do not respond to medical therapy and if OA is associated with impairments to their quality of life, several orthopaedic surgery options may be available. For example, total joint replacement is among the most effective of all medical interventions; the pain and disability of end-stage OA can be eliminated, restoring the patients to near-normal function [106].

In conclusion, a whole scale of symptom relieving drugs and therapies are available. However, today there are no drugs on hand which show sufficiently satisfactory disease modifying activity [107]. Hopefully, current and future research will provide new insights in this complex disease and will assist in the rational development of such drugs.



**Table 1:** Final set of 10 recommendations based on both evidence and expert opinion for the management of knee OA, proposed by the EULAR Standing Committee for International Clinical Studies Including Therapeutic Trials [105].

1	The optimal management of knee OA requires a combination of non-pharmacological and pharmacological treatment modalities
2	The treatment of knee OA should be tailored according to: (a) Knee risk factors (obesity, adverse mechanical factors, physical activity) (b) General risk factors (age, comorbidity, polypharmacy) (c) Level of pain intensity and disability (d) Sign of inflammation—for example, effusion (e) Location and degree of structural damage
3	Non-pharmacological treatment of knee OA should include regular education, exercise, appliances (sticks, insoles, knee bracing), and weight reduction
4	Paracetamol is the oral analgesic to try first and, if successful, the preferred long term oral analgesic
5	Topical applications (NSAID, capsaicin) have clinical efficacy and are safe
6	NSAIDs should be considered in patients unresponsive to paracetamol. In patients with an increased gastrointestinal risk, non-selective NSAIDs and effective gastroprotective agents, or selective COX 2 inhibitors should be used
7	Opioid analgesics, with or without paracetamol, are useful alternatives in patients in whom NSAIDs, including COX 2 selective inhibitors, are contraindicated, ineffective, and/or poorly tolerated
8	SYSADOA (glucosamine sulphate, chondroitin sulphate, ASU, diacerein, hyaluronic acid) have symptomatic effects and may modify structure
9	Intra-articular injection of long acting corticosteroid is indicated for flare of knee pain, especially if accompanied by effusion
10	Joint replacement has to be considered in patients with radiographic evidence of knee OA who have refractory pain and disability

### 3. Pathogenesis of OA: biomolecular perspective

#### 3.1 Cytokines and growth factors: the guardians of chondrocyte homeostasis?

As stated above, the matrix forms the functional component of the articular cartilage, whereas the chondrocytes are responsible for the homeostasis of this tissue. The extracellular matrix also forms the micro-environment for the chondrocytes, containing cytokines and growth

factors synthesized by the chondrocyte itself. In healthy adults, articular cartilage shows a slow tissue turnover and remodeling, based on an equal balance between anabolic and catabolic cytokines and growth factors. With respect to regulation of the chondrocyte function, it is possible to classify the cytokines that regulate cartilage remodeling as catabolic cytokines (which ultimately results in enhanced matrix degradation) and anabolic cytokines, (which act as growth and differentiation factors). In addition, several cytokines can be classified as modulatory cytokines, based on their capacity to modulate the activities of other cytokines, for example IL-6 and IL-11 (table 2) [108].

**Table 2:** The most prominent cytokines and growth factors involved in cartilage metabolism.

<b>Catabolic</b>	<b>Modulatory</b>	<b>Anabolic</b>
Interleukin-1 (IL-1)	Interleukin-6 (IL-6)	Insulin-like growth factor 1 (IGF-1)
Tumor necrosis factor $\alpha$ (TNF- $\alpha$ )	Interleukin-8 (IL-8)	Transforming growth factor $\beta$ (TGF- $\beta$ )
Interleukin-17 (IL-17)		Bone morphogenetic proteins (BMP) -2, 4, 6, 7, 9, 13
Interleukin-18 (IL-18)		Fibroblast growth factors (FGFs)

The best known catabolic cytokines are interleukin-1 (IL-1) and tumor-necrosis factor  $\alpha$  (TNF- $\alpha$ ). Studies, both in vitro and in vivo, show that the effects of IL-1 and TNF- $\alpha$  are similar or synergistic. It is well established that IL-1 contributes to the depletion of the cartilage matrix by decreasing the synthesis of cartilage-specific collagens and proteoglycans (reviewed in [108-110]). It is now generally accepted that these cytokines are involved in the matrix degradation associated with OA [108], as pathways associated with IL-1 and TNF- $\alpha$  seem to be activated in OA. Moreover, OA cartilage appears to be more susceptible to the degenerate effects of IL-1 [111] and TNF- $\alpha$ . For example, OA chondrocytes express more of the TNF-receptor than nonarthritic chondrocytes [112] and this receptor has been localized in cells at sites of focal loss of proteoglycans in OA cartilage [113]. On the other hand, an increased expression of the IL-1 receptor type I has been detected in OA chondrocytes in association with an enhanced production of matrix-metalloproteinases (MMPs) [114]. The MMPs are a family of proteases which play, together with the aggrecanases, a primary role in

the degradation of the extracellular matrix [115]. In recent years, a body of knowledge has become available concerning the mediators (such as NO and prostaglandins [116], matrix-metalloproteinases (MMPs) [117, 118]) and signal transduction pathways activated by these cytokines (for example nuclear factor- $\kappa$ B-mediated pathways [119, 120]). Other known, less characterized, catabolic cytokines in chondrocyte biology are IL-17 [121] and IL-18 [122].

Next to catabolic cytokines, there is a scale of anabolic factors which play an important role in both the preservation of normal articular cartilage and its failure in joint disease. Insulin-growth factor I (IGF-I) was among the first to be identified as an anabolic factor [123-125]. Transforming-growth factor  $\beta$  regulates a variety of cellular responses on the chondrocyte, via distinct pathways. For example, it has been shown to induce collagen type II expression in human articular chondrocytes [126]. However, the effects of TGF- $\beta$  on chondrocyte metabolism seem to be rather contradictory, given the observation that some groups reported catabolic effects of TGF- $\beta$  on cartilage (reviewed and discussed in [127]). Bone morphogenetic proteins (BMPs) are also considered as anabolic factors, especially BMP-2 and BMP-7, have been widely investigated and are known to maintain and promote the articular chondrocyte phenotype in vitro [128]. It has even been stated that no other growth factor equals the capacity of BMP-7 to produce a marked upregulation of the majority of ECM compounds [127]. Finally, a number of reports showed synergistic effects of different anabolic factors on chondrocytes [129-131].

In recent decades, several proteins have been elucidated which may influence the activity of these cytokines or their downstream “effectors” directly. In consequence, these proteins are essential to maintain the homeostasis in normal chondrocytes and may play a role in the pathogenesis of OA. Moreover, the expression of these modulators can be controlled by one or more of these cytokines or growth-factors. The action of IGF-1 on chondrocyte metabolism can be regulated by the presence of extracellular, high-affinity IGF-binding proteins (IGFBP), which modulate the interaction of IGF-1 with its receptor [132]. Alternatively, the IL-1 receptor II, which acts as a decoy receptor and whose expression is controlled by IGF-1, may reverse the catabolic activity of IL-1 on chondrocytes [133]. Another example are the tissue inhibitors of metalloproteinases (TIMPs): these act as inhibitors of MMPs. The expression of these inhibitors and binding proteins is also regulated by cytokines or growth factors [134-136], which may explain the very complex and very delicate balance between catabolic and anabolic processes in the chondrocyte.

### **3.2    *The role of the chondrocyte in the pathogenesis of OA***

The development of OA is associated with changes in the interterritorial matrix (more specifically changes in the collagen network and the interwoven proteoglycan aggregates), the cell-associated matrix (for example transformational conformation of resident type VI collagen) as well as in the cellular phenotype [137]. The chondrocyte has clinical importance in the context of OA pathogenesis, which results from a failure to maintain the balance between synthesis and degradation of the ECM (for a review see [138]). Therefore, it is generally believed that chondrocytes play important roles in the development of OA [139]. In early OA, the chondrocyte exhibits a transient proliferative response (clonal growth) [140], an increased synthesis of cartilage matrix in an attempt to repair cartilage breakdown [141, 142] and an increased expression of catabolic cytokines [143] and matrix-degrading enzymes such as matrix metalloproteinases [117] and aggrecanases [144] (reviewed in [145]). The cellular reaction during OA-development is pleiomorphic at different stages. First, next to an increased proliferation rate, it has also been suggested that cell death is a central feature in OA cartilage degeneration [146]. Apoptotic cell death has been reported as a major event in OA [147-149]. However, there are some contradictory viewpoints concerning the importance of apoptosis in the pathogenesis of OA [137]. Furthermore, despite an increased synthetic activity for extracellular matrix components in OA [142, 150], there is a net loss of functional cartilage matrix. It may be concluded that the OA-chondrocyte undergoes phenotypic alterations, which may represent a potential reason for the anabolic failure of chondrocytes in OA: despite the potential high synthetic activity, OA-chondrocytes may express other -less functional- genes. This changed phenotype ultimately results in the appearance of macroscopic phenomena such as fibrillations, matrix depletion, cell cluster formation and changes in quantity, distribution, or composition of matrix proteins [151].

In conclusion, it may be stated that the cellular activity of chondrocytes in OA is very complex and may vary during the course of the disease. As described above, a lot of knowledge is available at the biomolecular level. However, the big question remains: “What is/are the first step(s) in the initiation of cellular dysregulated activity in OA?” [139].

# **Chapter I: Introduction**

*Part 3: Proteomics: general principles and technical platforms*



Adapted from: **“Entering the Era of Proteomics in Rheumatology”**

Stijn Lambrecht<sup>1</sup>, Dieter Deforce<sup>1</sup>, Dirk Elewaut<sup>2</sup>

<sup>1</sup>Laboratory of Pharmaceutical Biotechnology, Ghent University, Belgium; <sup>2</sup>Laboratory for Molecular Immunology and Inflammation, Department of Rheumatology, Ghent University Hospital, Ghent, Belgium

**Expert Opinion on Drug Discovery, 2006, 1 (6), 539-48**

## Abstract

Proteomics, the large-scale analysis of proteins of a given cell or tissue, is a fast emerging field in biomedical research. It has become clear that proteomic approaches can assist in unraveling complex disease pathways and in the discovery of protein drug targets. The recent flood in proteomic-data demonstrates the furious digging in the quest for the so called “Holy Grail”. At present, however, only a limited number of reports describing proteomic studies in rheumatology have been published. This review highlights some recent advances in the field of proteomic techniques. These new techniques as well as classic approaches have potential applications in the field of rheumatology. There is little doubt that these and upcoming new proteomic tools will lead to landmark discoveries in this field with applications ranging from diagnostics, therapeutic monitoring to discovery of new therapeutic targets.



## 1. Introduction

The human genome sequencing has led to a revolution in molecular biology research. The analysis of the human genome introduced the large-scale, ‘-omics’ disciplines. Disciplines as genomics, transcriptomics, proteomics and metabo(l)/(n)omics have been developed in the recent decades and are characterized by an exponential growth over the past years. All these disciplines focus on a different level (DNA, mRNA, proteins, metabolites) in cell biology, making them complementary to each other. The insight that one gene can encode different protein isoforms made researchers realize that a genomics approach provides limited information in the global analysis of gene products. Several phenomena such as protein structure, protein modifications, protein function and protein-protein interactions, can only be studied at protein level. Proteomics is one of the fastest emerging fields in these ‘omics’ disciplines. An explanation for this flood of proteomic-data is that proteins are very attractive drug targets as they are the main executors of cellular function. Nowadays, almost all drugs have protein targets.

The general goal of proteomics can be described as the aim to obtain a more global and integrated view of biology by studying all the proteins of a cell rather than each one individually [152]. Three main areas can be discriminated in proteomics: the large-scale identification of proteins and post-translational modifications (PTMs), ‘differential display’ proteomics for comparison of protein levels in a wide range of diseases and studies of protein-protein interactions [153]. These three areas reveal different information and a combination of all is necessary to provide new insights in our understanding of biological processes.

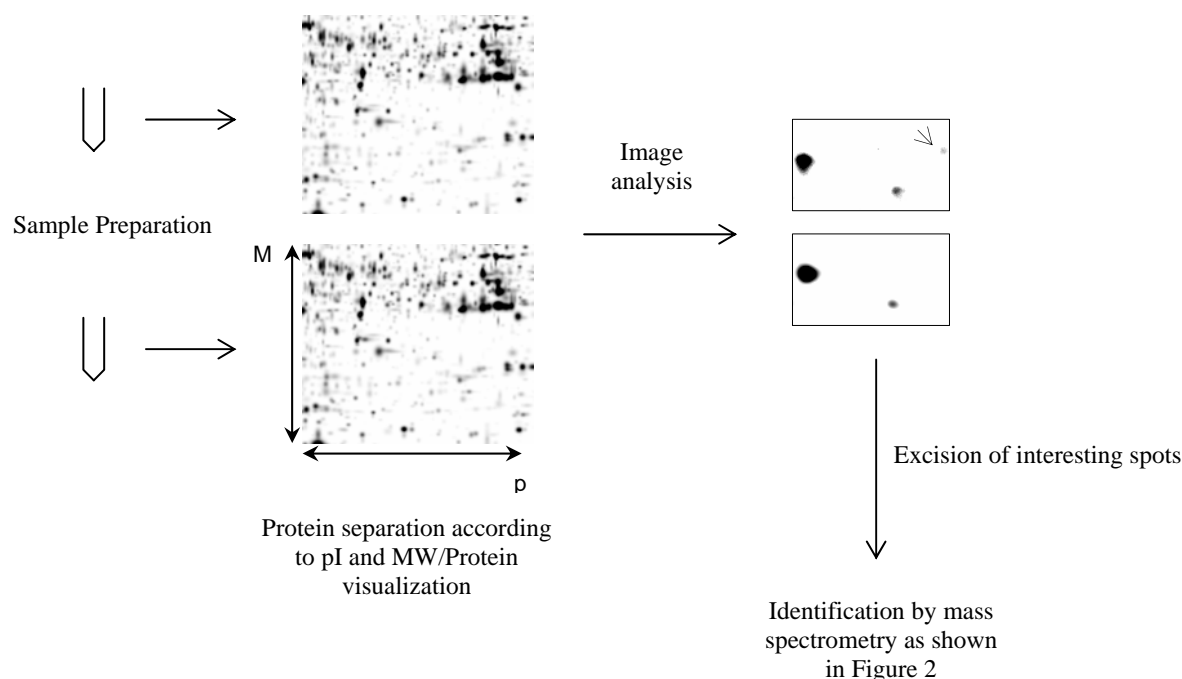
### ***1.1 The combination of ‘new’ and ‘established’ technologies opens up new perspectives***

A whole scale of techniques has become available to investigate the proteome of biological samples. For a detailed overview of the most prominent techniques in proteomics see reference [152]. In this review, we will focus on the most widely applied techniques in the field of rheumatology and recent advances will be highlighted.

#### **1.1.1 Two-dimensional gel electrophoresis**

Two-dimensional gel electrophoresis (2-DE) remains the most important tool of a classic proteomic approach. 2-DE was first described in 1975 by O’Farrell [154] and Klose [155]. It enables the separation of complex mixtures of proteins, based on the isoelectric point (pI) in the first dimension and on the molecular weight (MW) in the second dimension. A 2-DE

approach consists of several steps: sample preparation, protein separation, protein detection, image analysis and protein identification (Figure 6).



**Figure 6:** Overview of a classic two-dimensional gel approach for differential protein expression profiling. Proteins are extracted from cells (or tissues) in different conditions. The solubilized proteins are applied to a gel strip and separated according to their pI, which is usually performed in an immobilized pH gradient. After reduction and alkylation of cysteine residues, the strip is applied to a SDS-PAGE gel. The denatured proteins migrate in the applied electrical field and are separated based on their molecular weight. Gels are stained and imaged to visualize proteins. Protein spots are quantified and matched to each other with the appropriate software. Spots quantities are subjected to statistical analysis, resulting in a list of interesting protein spots. These spots can then be excised and subjected to mass spectrometry for identification.

The current state in 2-DE technology has been reviewed in [156]. Here, we will highlight some interesting and recent advances in this ‘old-fashioned’, but still ‘golden-standard’ technology.

One of the biggest challenges in proteome-analysis is the identification of lower abundant proteins, as these ‘hidden’ proteins may be the interesting ones (e.g. membrane receptors). Nowadays a wide range of sample preparation methods are applied resulting in higher dynamic ranges, the ability to resolve hydrophobic and acidic or basic proteins. Methods based on subcellular fractionation [157], depletion of highly abundant proteins [158], alternative solubilizing agents [159] and differential extraction [160] are widely used and have proven their efficiency. Such pre-fractionation methods aim to reduce the complexity of the sample and thereby revealing minor abundant proteins. Moreover, methods based on

subcellular fractionation provide interesting information about protein localization. It has to be noted that prefractionation methods based on the depletion of highly abundant proteins (e.g. albumin in serum samples), can result in the loss of low abundant proteins bound to these carrier proteins. As those may act as a reservoir for bound biomarkers [161], depletion methods may result in the loss of important information. For a detailed review on sample preparation methods in proteomics, see references [162] and [163].

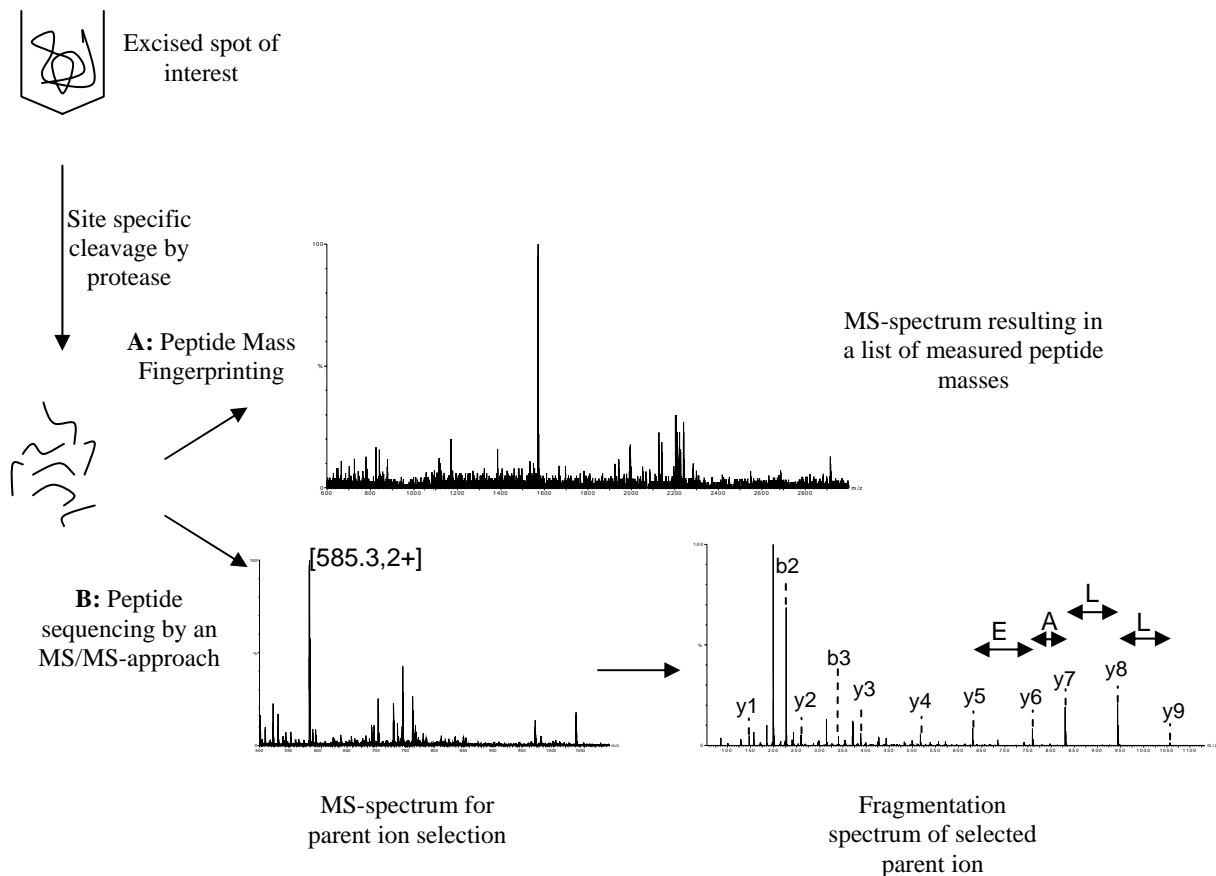
Since the introduction of 2-DE, tremendous improvements have been made. In particular, the introduction of immobilized pH gradients (IPG) [164], narrow-range pH IPG-strips [165] and fluorescent stains (reviewed in [166]) led to superior results in terms of resolution, sensitivity and reproducibility. For example, the linear dynamic range of Sypro Ruby reaches from 1-2 ng to 1-2 µg (8 to 60 ng for silver staining) [167]. Recently, sensitive fluorescent stains, which selectively stain phosphorylated [168] or glycosylated [169] proteins, have been introduced. The greatest power of 2-DE is the ability to resolve multiple isoforms of a protein, which differ in only a minor pI- or MW-value. As it is known that PTMs can influence protein activity, the use of a high-resolution 2-DE approach combined with the above mentioned fluorescent stains and recent advances in mass spectrometry (which will be discussed later in this review) can open a new era in the application of 2-DE.

As high accuracy in spot detection and matching is required to avoid artifacts, image analysis is a critical step in the '2-DE process'. The introduction of high-resolution scanners and specialized software facilitated spot detection, matching and quantification. Nevertheless, this step remains one of the most time-consuming steps in a classic proteomic approach. Advances herein, have been made by difference gel electrophoresis (DIGE) [170]. This technology is based on the fluorescent tagging of two protein samples with two different dyes (cyanine dyes: Cy3 and Cy5) prior to electrophoresis. The samples are mixed and can be run on the same gel. Fluorescence imaging of the gel is used to create separate images, which can be ideally superimposed. Moreover, a third color that represents an internal standard, a mixture of all samples labeled with Cy2, has been introduced to reduce inter-gel variability [171].

### **1.1.2 Mass spectrometry**

The application of mass spectrometry (MS) in protein analysis was a milestone in the field of proteomics. The discovery of 'soft' ionization techniques electrospray-ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) made it possible to analyze biomolecules. In a classic 2-DE approach, spots of interest are excised and treated with a proteolytic enzyme (e.g. trypsin) to produce a mixture of smaller peptides. The identification

of the unknown protein is based on the analysis of this peptide mixture (Figure 7). The detection limit of an MS-based approach is mainly limited by the sample preparation method. In general, for successful mass spectrometric identification of an in-gel digested protein, Sypro Ruby-stained amounts (low ng-range) are necessary. Using gel-free sample preparation methods (see 1.1.3) lower detection limits can be achieved. The most common technique to identify proteins is called peptide mass fingerprinting. As the peptide mixture is submitted to 'single MS' mass spectrometry (usually MALDI Time-Of-Flight (MALDI-TOF)), this yields a set of accurate mass-to-charge ratios of each peptide. This 'mass fingerprint' is then compared to theoretically expected peptide masses for each protein in a given protein sequence database. Alternatively, 'tandem MS' mass spectrometry (MS/MS) determines not only the mass of the peptide but yields also information about the amino acid sequence. In MS/MS mode a survey scan is performed after which a peptide is selected by the first mass analyzer and dissociated by collision with gas molecules, the so-called collision-induced dissociation (CID). This results in the fragmentation of the bonds along the peptide backbone. The tandem MS spectrum contains structural information related to the peptide sequence. Therefore, these searches are generally more specific than peptide mass fingerprinting. In contrast to peptide mass fingerprint data, these fragmentation data can also be submitted to nucleotide databases, such as expressed sequence tag databases (EST). Whether a single MS or a tandem MS approach is used, the identification is related to a certain score which is a measure for the level of confidence for the match between the spectra and theoretical sequence data. An extended description of MS in protein and peptide analysis is beyond the scope of this review, but is reviewed in detail in reference [172].



**Figure 7:** Strategies for mass spectrometric identification of protein spots of interest. After spots have been excised, proteins are digested. This is usually done by site specific proteases (e.g. trypsin cleavage results in peptides with arginine or lysine at the C-terminus). The resulting peptide mixture can be subjected to mass spectrometry. **A:** Using a Peptide Mass Fingerprinting (PMF) approach, the mass spectrometer (usually MALDI-TOF) generates a list of peaks indicating the mass-to-charge ratio of the peptides. This set of masses is compared with theoretically expected peptide masses for each protein in a database. Depending on the number of peptide matches, the search engine will return a score which is a measure for the level of confidence for the match. **B:** In a first step a survey scan is recorded by the mass spectrometer (usually ESI QqTOF). The resulting spectrum usually displays both singly and multiply charged species. A parent ion of interest is selected and subjected to collision-induced dissociation (CID). This results in a random dissociation of the peptide along the peptide backbones, yielding the so-called y and b ions. Mass differences between consecutive y or b ions are representative for a certain amino acid. Experimental MS/MS-spectra can be matched against theoretical MS/MS-spectra for the peptides present in a given database. Only a short 'sequence tag' can be sufficient to identify a certain protein.

### 1.1.3 'Gel-free' methods

As one of the major disadvantages of 2-DE is that high molecular weight proteins and extremely acidic or basic proteins are lost, methods were developed to omit 2-DE gels. In recent decades, chromatography- and MS-technologies have improved in a staggering way. Powerful chromatographic methods (based on liquid chromatography (LC)) and mass

spectrometry were successfully combined in the field of large-scale identification of proteins and their post-translational modifications. In the recent decade, multidimensional LC-MS/MS technologies were introduced. A whole cell lysate is digested chemically or by a protease. This very complex mixture of peptides is separated by multidimensional separation techniques and applied to MS/MS-analysis. These multidimensional separation techniques consist of a combination of size-exclusion, reversed-phase and/or ion-exchange chromatography. In this context, the group of Yates introduced the multidimensional protein identification technology (MudPIT), in which reversed-phase chromatography and strong cation-exchange chromatography were combined [173]. This allowed the identification of low-abundant proteins, such as transcription factors, demonstrating the possibility to identify proteins below the detection limit of a classic 2-DE approach. Such multidimensional approaches have also been used for large-scale protein phosphorylation analysis. Beausoleil et al [174] detected over 2000 phosphorylation sites in the nuclear fraction of HeLa cell lysates by a combination of strong-cation exchange chromatography and reverse-phase LC-MS/MS. Such large-scale analysis of protein phosphorylation can help to identify new kinase targets and provide new insights in signaling pathways.

Such approaches are interesting for the exploration of the proteome of a given cell-type or tissue. But, they do not allow direct quantification or comparison between different samples. Therefore, alternative strategies have been developed in the field of 'gel-free' differential protein expression profiling. Stable isotope labeling of samples allows for direct relative quantitative analysis by MS. The principle of these methods is based on the incorporation of a stable isotope in one of the two samples to be compared. As the measured peaks of the treated sample shifts with a known mass, a relative quantitative measurement is possible between the two samples, as determined by the peak ratio. Stable isotopes can be incorporated by incubation in a medium enriched with stable isotopes (e.g.,  $^{15}\text{N}$ ) [175], alternatively the incorporation can be done in vitro by protein or peptide derivatization. Isotope coded affinity tags (ICAT) allow relative changes to be determined by MS and is based on the attachment of an isotope label on cysteine [176]. A recently introduced and very promising method in quantitative gel-free proteomics is iTRAQ, which allow relative changes to be determined in MS/MS. This method is based on the incorporation of different isobaric mass labels (at the N-terminal and lysine side chains of peptides) in different samples. The reagents are differentially isotopically labeled such that a given derivatized peptide in all samples is isobaric and indistinguishable by chromatography, but yield different reporter ions in MS/MS (following CID) that can be used to identify and quantify individual samples [177]. One of the

major advantages of this technique to other labeling methods is that it does not increase complexity in MS-mode as the tags are isobaric. In combination with protein quantitation, labelling strategies may also be used to analyze protein processing by selective labeling of the N-terminus of the protein [178].

Another development in biomarker analysis is surface-enhanced laser desorption/ionization (SELDI-TOF-MS), which is especially useful in the analysis of the low-molecular weight proteome. Whereas the smallest proteins detected by 2-DE are about 10 to 15 kDa, SELDI enables the analysis of peptides and proteins ranging from 500 Da to 20 kDa and thereby the exploration of the low molecular weight proteome. This technology couples protein subspecies enrichment to presentation to the mass spectrometer. Using substrates with varying adsorptive properties (e.g., reversed phase resins, cation exchange resins, anion exchange resins...) (reviewed in [179]) and the application of various wash buffers, a fraction of the proteins can be selected. TOF-MS analysis results in spectra of complex protein mixtures based on the mass-to-charge ratio of the proteins. Statistical analysis of peak patterns from different disease states can yield multimarker patterns, which can be used to classify the samples. A major disadvantage of this approach is that it does not yield sequence information to identify the protein of interest. So, once the importance of a marker has been demonstrated, further purification, separation and digestion have to be applied for protein identification.

Finally, the development of array-based proteomic techniques is very promising in routine diagnostics as well as in biomarker discovery. Several array types can be distinguished (reviewed in [180]). Tissue microarrays (TMAs) are ordered arrays of tissue cores from multiple donor blocks, which allow the simultaneous analysis of numerous tissue samples with a detection system (e.g. an antibody). Forward-phase protein microarrays (FPAs) consist of several bound bait molecules (e.g. antibodies [181]) on the array's surface. Samples such as serum or synovial fluid are applied to these arrays and bound analytes can be visualized by direct labeling or by labeled antibodies. Finally, with Reverse-phase protein microarrays (RPAs) protein mixtures (e.g. cellular lysates) are spotted directly on the array and probed with specific antibodies [182]. The major power of these protein-arrays lies in the ability to screen for molecular networks. So, undoubtedly these arrays will contribute to future discoveries in signaling cascades associated with disease states.

#### **1.1.4 Protein interaction analysis**

To elucidate a biological function of a protein, the knowledge of its location and level of expression is not sufficient. The identification of the interaction partners of a protein is

indispensable to understand cell biology. Methods to study protein complexes are usually based on immunoprecipitation reactions. For example, the “bait” protein can be produced as a GST-fusion protein and then mixed with a cell lysate. Using GST-affinity purification, the “bait” protein and its interactors can be isolated and subsequently identified [183]. Alternatively, several other approaches are applicable, such as the yeast two-hybrid system [184], phage display and other MS-based approaches (reviewed in [185]). As the study of protein-interactions (‘interactomics’) has evolved to a specialized discipline, it will not be further discussed in this review.

Countless techniques and variations on these techniques have been developed in proteomics. As discussed above, every approach has its own advantages and disadvantages. The choice for a specific technique or a combination of different techniques, depends on the application and goal of the study. Whereas alternative (e.g. ‘gel-free’) methods are gaining field in the large-scale identification of proteins and their post-translational modifications, a 2-DE based approach remains the method of choice in many laboratories for comparison of protein levels in a set of several disease states.

One of the biggest challenges in the further development of proteomic techniques remains the identification of the low abundant proteins. For example, membrane receptors are very interesting drug targets, but they are only very rarely identified in a proteome study. Future optimization of current techniques and development of new strategies will probably enable further ‘digging’ in the human proteome.

## Acknowledgements

S. Lambrecht is a research assistant of the Fund for Scientific Research-Flanders (Belgium). D. Elewaut and D. Deforce are supported by grants of the Fund for Scientific Research-Flanders and the Research Council of Ghent University.



## References

- [1]Simkin, P. A., in: Klippel, J. H. (Ed.), *Primer on the rheumatic diseases*, Arthritis Foundation, Atlanta 2000, pp. 5-31.
- [2]Mankin, H. J. and Radin, E. L., in: Koopman, W. J. (Ed.), *Arthritis and allied conditions: A textbook of rheumatology*, Williams and Wilkins, Baltimore 1997, pp. 175-191.
- [3]Tarner, I. H., Harle, P., Muller-Ladner, U., Gay, R. E. and Gay, S., *Best Pract Res Clin Rheumatol* 2005, 19, 19-35.
- [4]Firestein, G. S., *Nature* 2003, 423, 356-361.
- [5]Smolen, J. S., Aletaha, D., Koeller, M., Weisman, M. H. and Emery, P., *Lancet* 2007.
- [6]Goldring, S. R., *Rheumatology (Oxford)* 2003, 42 Suppl 2, ii11-16.
- [7]Braun, J. and Sieper, J., *Lancet* 2007, 369, 1379-1390.
- [8]Ravelli, A. and Martini, A., *Lancet* 2007, 369, 767-778.
- [9]Pelletier, J. P., Martel-Pelletier, J. and Abramson, S. B., *Arthritis Rheum* 2001, 44, 1237-1247.
- [10]Dieppe, P. A. and Lohmander, L. S., *Lancet* 2005, 365, 965-973.
- [11]*Clin Pharmacol Ther* 2001, 69, 89-95.
- [12]Gough, A. K., Lilley, J., Eyre, S., Holder, R. L. and Emery, P., *Lancet* 1994, 344, 23-27.
- [13]Feldtkeller, E., Khan, M. A., van der Heijde, D., van der Linden, S. and Braun, J., *Rheumatol Int* 2003, 23, 61-66.
- [14]Felson, D. T., Lawrence, R. C., Hochberg, M. C., McAlindon, T., *et al.*, *Ann Intern Med* 2000, 133, 726-737.
- [15]Goldring, M. B., *Arthritis Rheum* 2000, 43, 1916-1926.
- [16]Masi, A. T., Maldonado-Cocco, J. A., Kaplan, S. B., Feigenbaum, S. L. and Chandler, R. W., *Semin Arthritis Rheum* 1976, 4, 299-326.
- [17]Bas, S., Perneger, T. V., Seitz, M., Tiercy, J. M., *et al.*, *Rheumatology (Oxford)* 2002, 41, 809-814.
- [18]Lisse, J. R., *Postgrad Med* 1993, 94, 133-134, 139.
- [19]Mewar, D. and Wilson, A. G., *Biomed Pharmacother* 2006, 60, 648-655.
- [20]Schellekens, G. A., de Jong, B. A., van den Hoogen, F. H., van de Putte, L. B. and van Venrooij, W. J., *J Clin Invest* 1998, 101, 273-281.
- [21]Bizzaro, N., Mazzanti, G., Tonutti, E., Villalta, D. and Tozzoli, R., *Clin Chem* 2001, 47, 1089-1093.
- [22]Nielen, M. M., van Schaardenburg, D., Reesink, H. W., van de Stadt, R. J., *et al.*, *Arthritis Rheum* 2004, 50, 380-386.
- [23]Rantapaa-Dahlqvist, S., de Jong, B. A., Berglin, E., Hallmans, G., *et al.*, *Arthritis Rheum* 2003, 48, 2741-2749.

- [24]Arita, K., Hashimoto, H., Shimizu, T., Nakashima, K., *et al.*, *Nat Struct Mol Biol* 2004, 11, 777-783.
- [25]Masson-Bessiere, C., Sebbag, M., Durieux, J. J., Nogueira, L., *et al.*, *Clin Exp Immunol* 2000, 119, 544-552.
- [26]Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.
- [27]Chapuy-Regaud, S., Sebbag, M., Baeten, D., Clavel, C., *et al.*, *J Immunol* 2005, 174, 5057-5064.
- [28]van Gaalen, F. A., van Aken, J., Huizinga, T. W., Schreuder, G. M., *et al.*, *Arthritis Rheum* 2004, 50, 2113-2121.
- [29]Chang, X. and Han, J., *Mol Carcinog* 2005.
- [30]Ishigami, A., Ohsawa, T., Hiratsuka, M., Taguchi, H., *et al.*, *J Neurosci Res* 2005, 80, 120-128.
- [31]Matsuo, K., Xiang, Y., Nakamura, H., Masuko, K., *et al.*, *Arthritis Res Ther* 2006, 8, R175.
- [32]Tilleman, K., Union, A., Cantaert, T., De Keyser, S., *et al.*, *Proteomics-Clinical Applications* 2007, 1, 32-64.
- [33]Ukaji, F., Kitajima, I., Kubo, T., Shimizu, C., *et al.*, *Ann Rheum Dis* 1999, 58, 169-174.
- [34]Saulot, V., Vittecoq, O., Charlionet, R., Fardellone, P., *et al.*, *Arthritis Rheum* 2002, 46, 1196-1201.
- [35]Kinloch, A., Tatzer, V., Wait, R., Peston, D., *et al.*, *Arthritis Res Ther* 2005, 7, R1421-1429.
- [36]Hueber, W., Kidd, B. A., Tomooka, B. H., Lee, B. J., *et al.*, *Arthritis Rheum* 2005, 52, 2645-2655.
- [37]Hueber, W., Tomooka, B. H., Zhao, X., Kidd, B. A., *et al.*, *Ann Rheum Dis* 2006.
- [38]Feng, Y., Ke, X., Ma, R., Chen, Y., *et al.*, *Clin Chem* 2004, 50, 416-422.
- [39]Yuan, G. H., Masuko-Hongo, K., Kato, T. and Nishioka, K., *Arthritis Rheum* 2003, 48, 602-611.
- [40]Xiang, Y., Sekine, T., Nakamura, H., Imajoh-Ohmi, S., *et al.*, *J Immunol* 2006, 176, 3196-3204.
- [41]Xiang, Y., Sekine, T., Nakamura, H., Imajoh-Ohmi, S., *et al.*, *Arthritis Rheum* 2004, 50, 1511-1521.
- [42]Li, Q. Z., Xie, C., Wu, T., Mackay, M., *et al.*, *J Clin Invest* 2005, 115, 3428-3439.
- [43]Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., *et al.*, *Electrophoresis* 2002, 23, 3445-3456.
- [44]Drynda, S., Ringel, B., Kekow, M., Kuhne, C., *et al.*, *Pathol Res Pract* 2004, 200, 165-171.
- [45]Dotzlaw, H., Schulz, M., Eggert, M. and Neeck, G., *Biochim Biophys Acta* 2004, 1696, 121-129.
- [46]Smith, M. A., Bains, S. K., Betts, J. C., Choy, E. H. and Zanders, E. D., *Clin Diagn Lab Immunol* 2001, 8, 105-111.
- [47]Yamagiwa, H., Sarkar, G., Charlesworth, M. C., McCormick, D. J. and Bolander, M. E., *J Orthop Sci* 2003, 8, 482-490.
- [48]Gibson, D. S., Blelock, S., Brockbank, S., Curry, J., *et al.*, *J Proteome Res* 2006, 5, 1988-1995.
- [49]Uchida, T., Fukawa, A., Uchida, M., Fujita, K. and Saito, K., *J Proteome Res* 2002, 1, 495-499.
- [50]Foell, D. and Roth, J., *Arthritis Rheum* 2004, 50, 3762-3771.
- [51]de Seny, D., Fillet, M., Meuwis, M. A., Geurts, P., *et al.*, *Arthritis Rheum* 2005, 52, 3801-3812.

- [52]Mosley, K., Tam, F. W., Edwards, R. J., Crozier, J., *et al.*, *Rheumatology (Oxford)* 2006, 45, 1497-1504.
- [53]Miyamae, T., Malehorn, D. E., Lemster, B., Mori, M., *et al.*, *Arthritis Res Ther* 2005, 7, R746-755.
- [54]Ryu, O. H., Atkinson, J. C., Hoehn, G. T., Illei, G. G. and Hart, T. C., *Rheumatology (Oxford)* 2006, 45, 1077-1086.
- [55]Tomosugi, N., Kitagawa, K., Takahashi, N., Sugai, S. and Ishikawa, I., *J Proteome Res* 2005, 4, 820-825.
- [56]Kuhn, E., Wu, J., Karl, J., Liao, H., *et al.*, *Proteomics* 2004, 4, 1175-1186.
- [57]Liao, H., Wu, J., Kuhn, E., Chin, W., *et al.*, *Arthritis Rheum* 2004, 50, 3792-3803.
- [58]Creamer, P. and Hochberg, M. C., *Lancet* 1997, 350, 503-508.
- [59]Appleton, C. T., Pitelka, V., Henry, J. and Beier, F., *Arthritis Rheum* 2007, 56, 1854-1868.
- [60]Wilkinson, L. S., Pitsillides, A. A., Worrall, J. G. and Edwards, J. C., *Arthritis Rheum* 1992, 35, 1179-1184.
- [61]Dasuri, K., Antonovici, M., Chen, K., Wong, K., *et al.*, *Arthritis Res Ther* 2004, 6, R161-168.
- [62]Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., *et al.*, *Proteomics* 2005, 5, 2247-2257.
- [63]Lorenz, P., Ruschpler, P., Koczan, D., Stiehl, P. and Thiesen, H. J., *Proteomics* 2003, 3, 991-1002.
- [64]Goldring, M. B., in: Klippel, J. H. (Ed.), *Primer on the rheumatic diseases*, Arthritis Foundation, Atlanta 2000, pp. 5-31.
- [65]Garcia, B. A., Platt, M. D., Born, T. L., Shabanowitz, J., *et al.*, *Rapid Commun Mass Spectrom* 2006, 20, 2999-3006.
- [66]Vincourt, J. B., Lionneton, F., Kratassiouk, G., Guillemin, F., *et al.*, *Mol Cell Proteomics* 2006, 5, 1984-1995.
- [67]Ruiz-Romero, C., Lopez-Armada, M. J. and Blanco, F. J., *Proteomics* 2005, 5, 3048-3059.
- [68]Ruiz-Romero, C., Lopez-Armada, M. J. and Blanco, F. J., *Osteoarthritis Cartilage* 2006, 14, 507-518.
- [69]Hermansson, M., Sawaji, Y., Bolton, M., Alexander, S., *et al.*, *J Biol Chem* 2004, 279, 43514-43521.
- [70]Lambrecht, S., Verbruggen, G., Verdonk, P., Elewaut, D. and Deforce, D., *Osteoarthritis Cartilage*, doi: 10.1016/j.joca.2007.06.005.
- [71]Villanueva, J., Philip, J., Chaparro, C. A., Li, Y., *et al.*, *J Proteome Res* 2005, 4, 1060-1072.
- [72]Senshu, T., Sato, T., Inoue, T., Akiyama, K. and Asaga, H., *Anal Biochem* 1992, 203, 94-100.
- [73]Muir, H., *Bioessays* 1995, 17, 1039-1048.
- [74]Martel-Pelletier, J., *Osteoarthritis Cartilage* 2004, 12 Suppl A, S31-33.
- [75]Bruckner, P. and van der Rest, M., *Microsc Res Tech* 1994, 28, 378-384.
- [76]Kuettner, K. E. and EJ, T., *Cartilage integrity and homeostasis.*, Dieppe P Kill J, London 1998, pp. 1-13.

- [77]Kuettner, K. E. and Goldberg, V. M., *Osteoarthritic disorders.*, Rosemont: American Academy of Orthopaedic Surgeons 1995.
- [78]Carmona, L., Ballina, J., Gabriel, R. and Laffon, A., *Ann Rheum Dis* 2001, 60, 1040-1045.
- [79]Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., *et al.*, *Ann Intern Med* 2000, 133, 635-646.
- [80]Le Pen, C., Reygrobelle, C. and Gerentes, I., *Joint Bone Spine* 2005, 72, 567-570.
- [81]Felson, D. T., *Am J Clin Nutr* 1996, 63, 430S-432S.
- [82]Arterburn, D. E., Crane, P. K. and Sullivan, S. D., *J Am Geriatr Soc* 2004, 52, 1907-1912.
- [83]Christensen, R., Bartels, E. M., Astrup, A. and Bliddal, H., *Ann Rheum Dis* 2007, 66, 433-439.
- [84]Loughlin, J., *Curr Opin Rheumatol* 2005, 17, 629-633.
- [85]Miyamoto, Y., Mabuchi, A., Shi, D., Kubo, T., *et al.*, *Nat Genet* 2007, 39, 529-533.
- [86]Mototani, H., Mabuchi, A., Saito, S., Fujioka, M., *et al.*, *Hum Mol Genet* 2005, 14, 1009-1017.
- [87]Kizawa, H., Kou, I., Iida, A., Sudo, A., *et al.*, *Nat Genet* 2005, 37, 138-144.
- [88]Valdes, A. M., Loughlin, J., Oene, M. V., Chapman, K., *et al.*, *Arthritis Rheum* 2007, 56, 137-146.
- [89]Loughlin, J., Sinsheimer, J. S., Carr, A. and Chapman, K., *Osteoarthritis Cartilage* 2006, 14, 295-298.
- [90]Forster, T., Chapman, K. and Loughlin, J., *Hum Genet* 2004, 114, 391-395.
- [91]Southam, L., Heath, O., Chapman, K. and Loughlin, J., *Ann Rheum Dis* 2006, 65, 556-557.
- [92]Goldring, S. R. and Goldring, M. B., *J Musculoskelet Neuronal Interact* 2006, 6, 376-378.
- [93]Eckstein, F., Cicuttini, F., Raynauld, J. P., Waterton, J. C. and Peterfy, C., *Osteoarthritis Cartilage* 2006, 14 Suppl A, A46-75.
- [94]Felson, D. T., *N Engl J Med* 2006, 354, 841-848.
- [95]Brandt, K. D., Radin, E. L., Dieppe, P. A. and van de Putte, L., *Ann Rheum Dis* 2006, 65, 1261-1264.
- [96]Eccles, M., Freemantle, N. and Mason, J., *Bmj* 1998, 317, 526-530.
- [97]Bradley, J. D., Brandt, K. D., Katz, B. P., Kalasinski, L. A. and Ryan, S. I., *N Engl J Med* 1991, 325, 87-91.
- [98]Williams, H. J., Ward, J. R., Egger, M. J., Neuner, R., *et al.*, *Arthritis Rheum* 1993, 36, 1196-1206.
- [99]*Arthritis Rheum* 2000, 43, 1905-1915.
- [100]Towheed, T. E., Maxwell, L., Anastassiades, T. P., Shea, B., *et al.*, *Cochrane Database Syst Rev* 2005, CD002946.
- [101]Poolsup, N., Suthisisang, C., Channark, P. and Kittikuluth, W., *Ann Pharmacother* 2005, 39, 1080-1087.
- [102]Clegg, D. O., Reda, D. J., Harris, C. L., Klein, M. A., *et al.*, *N Engl J Med* 2006, 354, 795-808.
- [103]Zhang, W., Doherty, M., Leeb, B. F., Alekseeva, L., *et al.*, *Ann Rheum Dis* 2007, 66, 377-388.
- [104]Zhang, W., Doherty, M., Arden, N., Bannwarth, B., *et al.*, *Ann Rheum Dis* 2005, 64, 669-681.

- [105]Jordan, K. M., Arden, N. K., Doherty, M., Bannwarth, B., *et al.*, *Ann Rheum Dis* 2003, 62, 1145-1155.
- [106]Lavernia, C. J., Guzman, J. F. and Gachupin-Garcia, A., *Clin Orthop Relat Res* 1997, 134-139.
- [107]Altman, R. D., *Semin Arthritis Rheum* 2005, 34, 3-5.
- [108]Goldring, S. R. and Goldring, M. B., *Clin Orthop Relat Res* 2004, S27-36.
- [109]Goldring, M. B., *Connect Tissue Res* 1999, 40, 1-11.
- [110]Westacott, C. I. and Sharif, M., *Semin Arthritis Rheum* 1996, 25, 254-272.
- [111]Ismail, S., Atkins, R. M., Pearse, M. F., Dieppe, P. A. and Elson, C. J., *Br J Rheumatol* 1992, 31, 369-373.
- [112]Westacott, C. I., Urban, J. P., Goldring, M. B. and Elson, C. J., *Biorheology* 2002, 39, 125-132.
- [113]Webb, G. R., Westacott, C. I. and Elson, C. J., *Osteoarthritis Cartilage* 1997, 5, 427-437.
- [114]Martel-Pelletier, J., McCollum, R., DiBattista, J., Faure, M. P., *et al.*, *Arthritis Rheum* 1992, 35, 530-540.
- [115]Martel-Pelletier, J. and Pelletier, J. P., *Semin Arthritis Rheum* 2005, 34, 6-8.
- [116]Goldring, M. B. and Berenbaum, F., *Clin Orthop Relat Res* 2004, S37-46.
- [117]Shlopov, B. V., Lie, W. R., Mainardi, C. L., Cole, A. A., *et al.*, *Arthritis Rheum* 1997, 40, 2065-2074.
- [118]Tetlow, L. C., Adlam, D. J. and Woolley, D. E., *Arthritis Rheum* 2001, 44, 585-594.
- [119]Ding, G. J., Fischer, P. A., Boltz, R. C., Schmidt, J. A., *et al.*, *J Biol Chem* 1998, 273, 28897-28905.
- [120]Mengshol, J. A., Vincenti, M. P., Coon, C. I., Barchowsky, A. and Brinckerhoff, C. E., *Arthritis Rheum* 2000, 43, 801-811.
- [121]Shalom-Barak, T., Quach, J. and Lotz, M., *J Biol Chem* 1998, 273, 27467-27473.
- [122]Olee, T., Hashimoto, S., Quach, J. and Lotz, M., *J Immunol* 1999, 162, 1096-1100.
- [123]Trippel, S. B., Corvol, M. T., Dumontier, M. F., Rappaport, R., *et al.*, *Pediatr Res* 1989, 25, 76-82.
- [124]Luyten, F. P., Hascall, V. C., Nissley, S. P., Morales, T. I. and Reddi, A. H., *Arch Biochem Biophys* 1988, 267, 416-425.
- [125]McQuillan, D. J., Handley, C. J., Campbell, M. A., Bolis, S., *et al.*, *Biochem J* 1986, 240, 423-430.
- [126]Frazer, A., Bunning, R. A., Thavarajah, M., Seid, J. M. and Russell, R. G., *Osteoarthritis Cartilage* 1994, 2, 235-245.
- [127]Chubinskaya, S. and Kuettner, K. E., *Int J Biochem Cell Biol* 2003, 35, 1323-1340.
- [128]Luyten, F. P., Yu, Y. M., Yanagishita, M., Vukicevic, S., *et al.*, *J Biol Chem* 1992, 267, 3691-3695.
- [129]Chubinskaya, S., Hakimiyan, A., Pacione, C., Yanke, A., *et al.*, *Osteoarthritis Cartilage* 2007, 15, 421-430.

- [130]Loeser, R. F., Pacione, C. A. and Chubinskaya, S., *Arthritis Rheum* 2003, 48, 2188-2196.
- [131]Yaeger, P. C., Masi, T. L., de-Ortiz, J. L., Binette, F., *et al.*, *Exp Cell Res* 1997, 237, 318-325.
- [132]Dore, S., Pelletier, J. P., DiBattista, J. A., Tardif, G., *et al.*, *Arthritis Rheum* 1994, 37, 253-263.
- [133]Wang, J., Elewaut, D., Veys, E. M. and Verbruggen, G., *Arthritis Rheum* 2003, 48, 1281-1291.
- [134]Matsumoto, T., Tsukazaki, T., Enomoto, H., Iwasaki, K. and Yamashita, S., *Ann Rheum Dis* 1994, 53, 128-133.
- [135]Silacci, P., Dayer, J. M., Desgeorges, A., Peter, R., *et al.*, *J Biol Chem* 1998, 273, 13625-13629.
- [136]Gunther, M., Haubeck, H. D., van de Leur, E., Blaser, J., *et al.*, *Arthritis Rheum* 1994, 37, 395-405.
- [137]Aigner, T. and McKenna, L., *Cell Mol Life Sci* 2002, 59, 5-18.
- [138]Goldring, M. B., *Best Pract Res Clin Rheumatol* 2006, 20, 1003-1025.
- [139]Aigner, T., Kurz, B., Fukui, N. and Sandell, L., *Curr Opin Rheumatol* 2002, 14, 578-584.
- [140]Hulth, A., Lindberg, L. and Telhag, H., *Clin Orthop Relat Res* 1972, 84, 197-199.
- [141]Lippiello, L., Hall, D. and Mankin, H. J., *J Clin Invest* 1977, 59, 593-600.
- [142]Ryu, J., Treadwell, B. V. and Mankin, H. J., *Arthritis Rheum* 1984, 27, 49-57.
- [143]Moos, V., Fickert, S., Muller, B., Weber, U. and Sieper, J., *J Rheumatol* 1999, 26, 870-879.
- [144]Lohmander, L. S., Neame, P. J. and Sandy, J. D., *Arthritis Rheum* 1993, 36, 1214-1222.
- [145]Caterson, B., Flannery, C. R., Hughes, C. E. and Little, C. B., *Matrix Biol* 2000, 19, 333-344.
- [146]Vignon, E., Arlot, M., Patricot, L. M. and Vignon, G., *Clin Orthop Relat Res* 1976, 303-308.
- [147]Blanco, F. J., Guitian, R., Vazquez-Martul, E., de Toro, F. J. and Galdo, F., *Arthritis Rheum* 1998, 41, 284-289.
- [148]Hashimoto, S., Ochs, R. L., Komiya, S. and Lotz, M., *Arthritis Rheum* 1998, 41, 1632-1638.
- [149]Kim, H. A., Lee, Y. J., Seong, S. C., Choe, K. W. and Song, Y. W., *J Rheumatol* 2000, 27, 455-462.
- [150]Collins, D. H. and Mc, E. T., *Ann Rheum Dis* 1960, 19, 318-330.
- [151]Pritzker, K. P., Gay, S., Jimenez, S. A., Ostergaard, K., *et al.*, *Osteoarthritis Cartilage* 2006, 14, 13-29.
- [152]Graves, P. R. and Haystead, T. A., *Microbiol Mol Biol Rev* 2002, 66, 39-63; table of contents.
- [153]Pandey, A. and Mann, M., *Nature* 2000, 405, 837-846.
- [154]O'Farrell, P. H., *J Biol Chem* 1975, 250, 4007-4021.
- [155]Klose, J., *Humangenetik* 1975, 26, 231-243.
- [156]Gorg, A., Weiss, W. and Dunn, M. J., *Proteomics* 2004, 4, 3665-3685.
- [157]Taylor, R. S., Wu, C. C., Hays, L. G., Eng, J. K., *et al.*, *Electrophoresis* 2000, 21, 3441-3459.
- [158]Bjorhall, K., Miliotis, T. and Davidsson, P., *Proteomics* 2005, 5, 307-317.
- [159]Rabilloud, T., *Electrophoresis* 1998, 19, 758-760.
- [160]Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., *et al.*, *Electrophoresis* 1998, 19, 837-844.

- [161]Mehta, A. I., Ross, S., Lowenthal, M. S., Fusaro, V., *et al.*, *Dis Markers* 2003, 19, 1-10.
- [162]Righetti, P., Castagna, A., Antonioli, P. and Boschetti, E., *Electrophoresis* 2005, 26, 297-319.
- [163]Shaw, M. M. and Riederer, B. M., *Proteomics* 2003, 3, 1408-1417.
- [164]Bjellqvist, B., Pasquali, C., Ravier, F., Sanchez, J. C. and Hochstrasser, D., *Electrophoresis* 1993, 14, 1357-1365.
- [165]Gorg, A., Obermaier, C., Boguth, G., Harder, A., *et al.*, *Electrophoresis* 2000, 21, 1037-1053.
- [166]Patton, W. F., *Electrophoresis* 2000, 21, 1123-1144.
- [167]Berggren, K., Chernokalskaya, E., Steinberg, T. H., Kemper, C., *et al.*, *Electrophoresis* 2000, 21, 2509-2521.
- [168]Schulenberg, B., Aggeler, R., Beechem, J. M., Capaldi, R. A. and Patton, W. F., *J Biol Chem* 2003, 278, 27251-27255.
- [169]Steinberg, T. H., Pretty On Top, K., Berggren, K. N., Kemper, C., *et al.*, *Proteomics* 2001, 1, 841-855.
- [170]Unlu, M., Morgan, M. E. and Minden, J. S., *Electrophoresis* 1997, 18, 2071-2077.
- [171]Alban, A., David, S. O., Bjorkesten, L., Andersson, C., *et al.*, *Proteomics* 2003, 3, 36-44.
- [172]Mann, M., Hendrickson, R. C. and Pandey, A., *Annu Rev Biochem* 2001, 70, 437-473.
- [173]Washburn, M., Wolters, D. and Yates, J., *Nat Biotechnol* 2001, 19, 242-247.
- [174]Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., *et al.*, *Proc Natl Acad Sci U S A* 2004, 101, 12130-12135.
- [175]Krijgsveld, J., Ketting, R., Mahmoudi, T., Johansen, J., *et al.*, *Nat Biotechnol* 2003, 21, 927-931.
- [176]Gygi, S., Rist, B., Gerber, S., Turecek, F., *et al.*, *Nat Biotechnol* 1999, 17, 994-999.
- [177]Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., *et al.*, *Mol Cell Proteomics* 2004, 3, 1154-1169.
- [178]Gevaert, K., Goethals, M., Martens, L., Van Damme, J., *et al.*, *Nat Biotechnol* 2003, 21, 566-569.
- [179]Weinberger, S. R., Dalmaso, E. A. and Fung, E. T., *Curr Opin Chem Biol* 2002, 6, 86-91.
- [180]Gulmann, C., Sheehan, K. M., Kay, E. W., Liotta, L. A. and Petricoin, E. F., 3rd, *J Pathol* 2006, 208, 595-606.
- [181]Haab, B. B., *Mol Cell Proteomics* 2005, 4, 377-383.
- [182]Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L., *et al.*, *Oncogene* 2001, 20, 1981-1989.
- [183]Joberty, G., Perlungher, R., Sheffield, P., Kinoshita, M., *et al.*, *NAT CELL BIOL* 2001, 3, 861-866.
- [184]Fields, S. and Song, O., *Nature* 1989, 340, 245-246.
- [185]Borch, J., Jorgensen, T. J. and Roepstorff, P., *Curr Opin Chem Biol* 2005, 9, 509-516.





## **Chapter II: Aim and outline of the study**

Osteoarthritis is the most common disabling condition in the western world. Given current life-style and future demographic evolutions, this disease is and will remain to be a major health problem [1]. OA is not directly a life-threatening disease. However, it has major impact both on the quality of life of the individual patient and on the economy [2].

A major hallmark of osteoarthritis is the slowly progressive degeneration of the articular cartilage, ultimately leading to distortion of other joint structures. In recent years, it has become clear that chondrocytes, the only resident cells in articular cartilage, play a central role in the initiation and/or the progression of the disease. As described throughout this study a lot of research efforts have already been conducted to study the physiopathology of chondrocytes in OA. The boom of biomolecular research in the recent decades is also reflected in this domain. However, at this time no sufficiently satisfactory disease-modifying drugs are available in the management of OA. A better understanding of underlying pathogenic processes may be crucial in the rational development of such drugs.

The primary goal of this study was to identify differentially expressed or abundant proteins between chondrocytes isolated from ‘normal’ and OA-patients. In recent years, gel-free proteomics techniques have been introduced in differential expression analyses. However, as previously stated in the introduction, a two-dimensional gel electrophoresis approach remains a very attractive approach to screen for differentially expressed proteins or protein isoforms. Proteome analyses, especially gel-based approaches, enable the identification of post-translational modifications, such as protein cleavage. In our study, protein patterns of different patient groups were compared by 2-D PAGE and potential interesting protein spots were identified by mass spectrometry. The differential abundance of a given spot, does not necessarily indicate that the corresponding gene is differentially expressed. This was clearly reflected in the subsequent validation of two spots identified as vimentin (*Chapter III, Part 1*). These turned out to be cleavage fragments, indicative of cytoskeletal disarrangements.

While the identification of a group of differentially abundant protein spots may provide new insights, it remains mandatory to perform additional validation studies to assess the biological importance of a potential interesting protein. In the second part of this study, we focussed on the potential role of  $\alpha$ Bcrystallin in the chondrocyte’s biology. Advances in mass spectrometry equipment, enabled us to reliably identify this differentially expressed protein spot discovered in the first study. Next to the quest for potential inducers/suppressors of this

protein, we investigated its relation with chondrocyte matrix-genes using RNA-interference (*Chapter III, Part 2*).

In recent years, studies have been initiated to disclose the proteome of human chondrocytes and cartilage [3-6]. Despite these studies, comprehensive information of the chondrocyte proteome remains limited. Especially the membrane and low-abundance proteome remain largely unknown. In the third part of this study, the aim was to further explore the proteome expressed by human knee chondrocytes, providing novel leads for future biomolecular research (*Chapter III, Part 3*).

All together, this study presents an introduction of proteomics technologies in OA-research at the chondrocyte level. The 2-DE analysis provided a platform to identify potentially interesting processes associated with OA. By validating the results of such proteome analyses with independent and complementary techniques, we aimed to present some candidate proteins which may shed a new light on chondrocyte biology and/or OA-pathogenesis.

## References

- [1] Creamer, P., Hochberg, M. C., Osteoarthritis. *Lancet* 1997, 350, 503-508.
- [2] Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., *et al.*, Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Ann Intern Med* 2000, 133, 635-646.
- [3] Vincourt, J. B., Lionneton, F., Kratassiouk, G., Guillemin, F., *et al.*, Establishment of a reliable method for direct proteome characterization of human articular cartilage. *Mol Cell Proteomics* 2006, 5, 1984-1995.
- [4] Garcia, B. A., Platt, M. D., Born, T. L., Shabanowitz, J., *et al.*, Protein profile of osteoarthritic human articular cartilage using tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2006, 20, 2999-3006.
- [5] Ruiz-Romero, C., Lopez-Armada, M. J., Blanco, F. J., Proteomic characterization of human normal articular chondrocytes: a novel tool for the study of osteoarthritis and other rheumatic diseases. *Proteomics* 2005, 5, 3048-3059.
- [6] Ruiz-Romero, C., Lopez-Armada, M. J., Blanco, F. J., Mitochondrial proteomic characterization of human normal articular chondrocytes. *Osteoarthritis Cartilage* 2006, 14, 507-518.



## **Chapter III: Results**

*Part 1: Differential proteome analysis of normal and osteoarthritic chondrocytes reveals distortion of vimentin network in osteoarthritis*



## **Differential proteome analysis of normal and osteoarthritic chondrocytes reveals distortion of vimentin network in osteoarthritis**

Stijn Lambrecht<sup>1</sup>, Gust Verbruggen<sup>3</sup>, MD, PhD, Peter C.M. Verdonk<sup>2</sup>, MD, PhD, Dirk Elewaut<sup>3</sup>, MD, PhD, Dieter Deforce<sup>1</sup>, PhD

<sup>1</sup>Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium; <sup>2</sup>Department of Orthopaedic Surgery, University Hospital Ghent, B-9000 Ghent, Belgium; <sup>3</sup>Department of Rheumatology, University Hospital Ghent, B-9000 Ghent, Belgium

**Corresponding author:** Prof. Dieter Deforce; Laboratory of Pharmaceutical Biotechnology, Harelbekestraat 72, B-9000 Ghent, Belgium; e-mail: Dieter.Deforce@Ugent.be; Fax: +32-9-220-6688.

S. Lambrecht and P.C.M. Verdonk are supported by grants of the Fund for Scientific Research-Flanders (Belgium).

**Accepted for publication: Osteoarthritis and Cartilage, 2007,  
doi:10.1016/j.joca.2007.06.005**

## Abstract

*Objective:* We conducted a proteome analysis of human articular chondrocytes, in order to identify proteins differentially expressed in chondrocytes during the progression of osteoarthritis (OA) and to characterize the phosphorylation status of these proteins.

*Methods:* The proteins of 20 samples of human chondrocytes obtained from the cartilage of human knees (6 from healthy cartilage (NoNo), 7 from visually intact zones (NoOA) and 7 from visually damaged zones (OAOA) of OA-cartilage from the same knee joint) were sequentially extracted and subjected to two-dimensional gel electrophoresis. Protein expression patterns were subjected to statistical analysis and protein spots of interest were identified by electrospray ionization tandem mass spectrometry.

*Results:* We identified several protein spots, showing a differential expression between the sample groups. Cleaved vimentin was upregulated in OAOA samples, this was confirmed by 1-D electrophoresis and Western blot. The possible impact of vimentin cleavage on the chondrocyte's cytoskeleton was illustrated by confocal microscopy analysis, which revealed a distorted vimentin organisation in OA chondrocytes. In contrast, F-actin staining did not reveal differences.

*Conclusion:* All together, this study revealed substantial alterations in the vimentin cytoskeleton in OA-affected human articular chondrocytes.



## 1. Introduction

Osteoarthritis (OA) is the most common disabling human condition in the western world, which ultimately results in degeneration of the articular cartilage. Now, it is clear that the chondrocyte plays a critical role in cartilage degeneration. In the recent years, several studies were performed to reveal changes in the cellular phenotype during OA-cartilage degeneration. Some of them investigated the expression of single genes by RT-PCR, Western blotting or immunocyto- and histochemistry [1-5]. Other studies investigated a broader set of genes using cDNA-array technology [6] or antibody microarrays [7]. In a recent study, the gene expression profiles of normal cartilage and intact and damaged regions of OA-cartilage were compared using cDNA arrays [8]. Despite these studies, the knowledge about the molecular mechanism of cartilage destruction in OA can still be improved.

The introduction of proteomics techniques enables the large-scale identification of proteins and the large-scale comparison of protein levels in a wide range of diseases. A whole scale of techniques has become available to elucidate the human proteome. Nevertheless, two-dimensional gel electrophoresis (2-DE) is the most widely applied technique to screen for differentially expressed proteins. 2-DE can still be considered as the most comprehensive analysis method for detecting a wide variety of post-translational modifications (PTMs), for example phosphorylation and protein cleavage. In such an approach, proteins are separated based on the isoelectric point (pI) in the first dimension and on the molecular weight (Mw) in the second dimension. After spot visualization, spot intensities can be compared and spots of interest can be identified by mass spectrometry (MS). Recently, some studies describing the proteome of chondrocytes [9] or cartilage [10] have been published. However, no comparison was made between healthy and OA-affected chondrocytes. Using a 2-DE approach, Hermansson *et al.* [11] investigated the proteins secreted by normal and OA-chondrocytes. This study revealed several differentially secreted proteins by OA-chondrocytes, but intracellular proteins were not investigated. To gain new insights in the molecular control mechanisms, involved in the development of OA, we analyzed the proteome of cultured chondrocytes and their cell associated matrix (CAM) from healthy donors and OA-patients. Since chondrocytes are the only cells in cartilage, which regulate the normal anabolism and catabolism of the cartilage matrix, an alteration in the protein expression pattern of these cells can influence the normal matrix homeostasis. The identification of differentially expressed

proteins can help us to identify new players in the progression of OA, to better understand the underlying disease-processes and to define novel therapeutic targets in this disease.

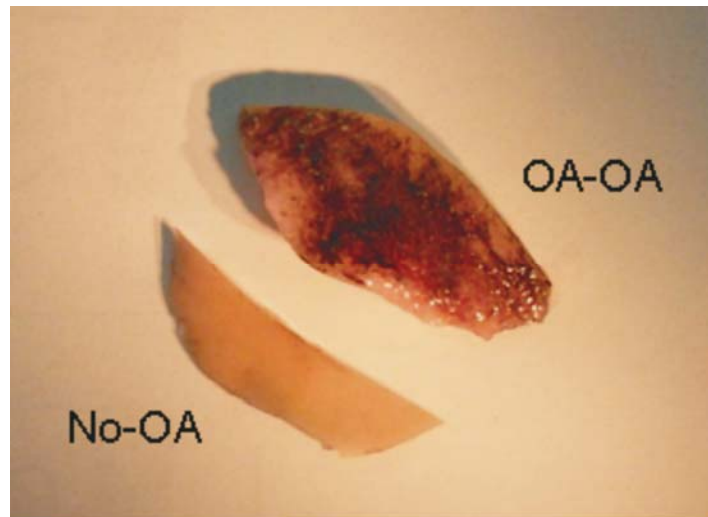
Aigner *et al.* [12] reviewed studies of functional genomics of OA and emphasized the importance of such gene expression analysis to evaluate disease hypotheses and understand complex diseases such as OA. However, mRNA levels often do not correlate with protein levels and phenomena such as post-translational modifications (PTMs) and protein-processing can not be discovered using such transcriptome studies. A proteomic approach, which identifies post-translational modifications, differentially expressed proteins or differentially processed proteins is a valuable complement to gene-expression studies.

Using two-dimensional electrophoresis, sequential staining procedures and mass spectrometry, we were able to identify isoforms of proteins showing an altered abundance in chondrocytes isolated from healthy cartilage versus visually intact or degenerated zones of OA-cartilage. The combination of these data with Western blot analysis and confocal microscopy revealed a distorted vimentin organization in OA chondrocytes.

## 2. Materials and Methods

### 2.1 Subjects

Healthy articular cartilage (NoNo) was obtained from 12 donors (9 male, 3 female; ages 18-72 years) within 24 h post-mortem. All donors had died as a result of trauma or a brief illness and none of them had been receiving corticosteroids or cytostatic drugs. OA affected cartilage was obtained from 14 patients (6 male, 8 female; ages 47-79 years) within 24 h from total knee arthroplasty. The cartilage from each of these patients was separated in visually intact cartilage (NoOA) and cartilage showing OA-lesions (OAOA). Figure 1 shows an image of a sample of NoOA and OAOA cartilage from the same patient, stained with indian ink, allowing fibrillated cartilage to be recognized [13]. The study was approved by the local Ethics Committee.



**Figure 1:** Image of articular cartilage stained with Indian ink. Both samples were isolated from the femur condyle from the same patient. On the upper slice (OAOA) the fibrillated surface is clearly visible. The lower slice (NoOA) shows a smooth, non-fibrillated surface.

## 2.2 *Isolation and culture of chondrocytes*

Human articular chondrocytes were isolated as previously described [14]. The cartilage was diced into small fragments and chondrocytes were isolated by sequential enzymatic digestion (hyaluronidase, pronase, collagenase (Sigma-Aldrich, Steinheim, Germany)) as described in detail elsewhere [14]. Trypan blue exclusion revealed that >95% of the cells were viable after isolation.

Chondrocyte cultures in alginate beads were prepared as described by Guo et al [15], with some modifications [16]. Briefly, chondrocytes suspended in 1 volume of double-concentrated Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY, USA) without calcium and magnesium, were carefully mixed with an equal volume of 2% alginate. The final chondrocyte concentration was  $5 \times 10^6$  cells/ml. The chondrocyte-alginate suspension was slowly dripped through a 23-gauge needle into a 102 mM solution of calcium chloride and the beads were allowed to polymerize for 10 minutes at room temperature. Calcium chloride was removed and the beads were washed 3 times with 0,9 % sodium chloride.

The beads were maintained in a 6-well plate (20 beads/well;  $\pm 50.000$  chondrocytes/bead) containing DMEM (Gibco) with 10% fetal calf serum, antibiotics and antimycotics (Gibco) and 50  $\mu\text{g/ml}$  ascorbate in an incubator at 37°C and in 5%  $\text{CO}_2$ . Medium was replaced three times a week for 10 days.

### 2.3 *Two-dimensional PAGE*

After the culture period, the medium was aspirated and the alginate beads were washed and dissolved by incubation in 55 mM tri-sodium citrate dihydrate pH 6.8, at room temperature. The resulting suspension was centrifuged at 1500 rpm for 10 min to separate cells with their CAM [17] from the constituents of the interterritorial matrix [5]. The resulting cell-pellet was washed three times with PBS. After washing and centrifugation, the chondrocytes were resuspended in Reagent 1 (40mM Tris) from the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA), containing protease inhibitors (Roche Diagnostics, Mannheim, Germany), a phosphatase inhibitor-cocktail (Sigma-Aldrich, Steinheim, Germany) and endonucleases (Sigma-Aldrich). Cells were lysed and the proteins were extracted using the ReadyPrep Sequential Extraction Kit (Bio-Rad) according to the manufacturer's protocol with the exception that the second and third fraction were combined. This resulted in a soluble and hydrophobic protein fraction. Using this approach an increase in the total number of protein spots is detected and a more simplified protein pattern is achieved [18]. The soluble fraction is considered to be the representative of the cytosolic proteins and the hydrophobic fraction of the membrane-associated proteins.

Two-dimensional electrophoresis (2-DE) was performed according to Tilleman et al. [19] with some minor modifications. The soluble fraction was desalted using ice-cold acetone precipitation. Proteins were dissolved in 300  $\mu$ l of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 0,2% carrier ampholyte solution and 12  $\mu$ l/ml Destreak Reagent (Amersham Biosciences, Uppsala, Sweden). The hydrophobic fraction was diluted with an appropriate volume of the above rehydration buffer to achieve a final volume of 300  $\mu$ l. Samples were applied to 17 cm non-linear pH 3-10 strips (Bio-Rad) and rehydrated overnight. Isoelectric focusing was performed for 100 kVh on the Protean Iso-Electrical-Focusing (IEF) cell (Bio-Rad). After focusing, proteins were reduced and alkylated by incubating the strips for 15 min in 15 ml of equilibration buffer (6 M urea, 2 % (w/v) sodium-dodecyl-sulfate (SDS), 50 mM TrisHCl, 30% glycerol) containing 2% dithiothreitol (DTT) (w/v) for reduction and 4% (w/v) iodoacetamide for alkylation respectively.

The strips were transferred onto vertical 12% (soluble fraction) and 10% (hydrophobic fraction) self-cast gels and fixed in place with 0,5% agarose. The second-dimensional gel electrophoresis was performed on a vertical Protean II xi Multi Cell (Bio-Rad) for 30 min at 16mA/gel followed by 32 mA/gel for approximately 4.5 h at 10°C.

The gels were sequentially stained with Pro-Q Diamond (Molecular Probes, Eugene, Oregon) according to the manufacturer's protocol. PeppermintStick Phosphoprotein Molecular Weight

Standards (Molecular Probes) were run beside the IEF-strip as positive and negative control for the Pro-Q Diamond stain. The image was acquired using a VersaDoc imaging system (Bio-Rad) with UV-excitation and a 520 nm longpass emission filter. After detection of the phospho-image, gels were stained with Sypro Ruby (Bio-Rad) according to the manufacturer's protocol. Proteins were detected using a VersaDoc with UV-excitation and a 520 nm longpass emission filter.

Sypro Ruby stained images were processed and analyzed using PDQuest software Version 7.10 (Bio-Rad). Spots were detected using the spot detection wizard, background was removed by applying the floater background. Gels were matched to each other creating a match set standard image, which contains match information from all the individual gels. Spot intensities were normalized for total quantity in valid spots, in order to correct for small pipetting errors, minor staining differences, etc. This normalization strategy implies that the raw quantity of each spot is divided by the total quantity of all the spots in that gel that have been included in the master. Spots were considered as 'significant differentially expressed' if spot intensities were significant ( $p$ -value  $<0.05$ ) different between the groups NoNo and NoOA or OAOA (Mann-Whitney U-test). The 'NoOA' and 'OAOA' groups were compared using Wilcoxon's paired-sample test;  $p$ -values less than 0.05 were considered statistically significant.

## **2.4 Sample preparation for MS analysis**

2D gel spots of interest were excised and transferred to micro-centrifuge tubes. The gel spots were washed twice (10 minutes/wash) with 200  $\mu$ l of 25 mM ammonium bicarbonate/50% acetonitrile, dehydrated using acetonitrile until spots turn opaque and dried until complete dryness in a vacuum centrifuge. Proteins were digested with sequencing grade trypsin (Promega, Madison, WI., 10ng/ $\mu$ l in 25 mM ammonium bicarbonate) overnight at 37°C. Peptides were extracted twice using, 75 $\mu$ l of a 50% acetonitrile/5% trifluoroacetic acid solution, extracts of the same spot were pooled and dried in a vacuum centrifuge.

## **2.5 Mass spectrometry and database analysis**

Peptides were resuspended using 0,1 % formic acid. For electrospray-ionization quadrupole time-of-flight (ESI Qq-Tof) analysis the resuspended peptides were injected on a Famos autosampler (LC Packings, Sunnyvale, CA, USA) and concentrated on a trap-column (PepMap, LC Packings) in buffer A (0,1% formic acid in water). Peptides were separated on a C18 PepMap column (LC Packings) by a 45 min linear gradient from 6 to 100 % buffer B

(80% acetonitrile and 0,1% formic acid in water). The flow rate was 150 nl/min (Ultimate pump, LC Packings).

ESI Qq-ToF analysis was performed on a Q-ToF I or a Q-ToF Ultima mass spectrometer (Waters, Milford USA), which was coupled to the LC-system via a nano-LC inlet. The instrument was calibrated using fragment ions generated from tandem mass-spectrometry (MS/MS) spectra of Glu-fibrinopeptide B (Sigma-Aldrich).

Data processing and database searches against the Swiss-Prot database were performed using the Protein Lynx Global Server V.2.0 search engine (Waters) and via Mascot (<http://www.matrixscience.com>) or GPM X!3 algorithm ([www.thegpm.org](http://www.thegpm.org)) after processing the MS/MS spectra using Mass Lynx v4.0. For an extended description of the protein identification parameters and the validation of the identifications, see supplementary data.

## **2.6 Western Blot analysis**

Equal amounts (30 µg as determined by 2-D Quant kit, GE Healthcare, Fairfield, USA) of soluble fractions of 12 patients (4 NoNo, 4 NoOA and 4 OAOA) were loaded on 10% SDS-PAGE gel. Equal loading was verified by Ponceau S staining (data not shown). MagicMark (Invitrogen, Paisley, UK) protein standards were run as molecular weight markers. 2-D gels were run as described above (soluble fraction from 2 NoNo samples and 2 OAOA samples), except that sample was applied to IPG-zoom strips (pH 4-7, Bio-Rad) and, after equilibration, strips were transferred to precast 10% SDS-PAGE Criterion gels (Bio-Rad). Strips were focused for 35 kVh and SDS-PAGE was performed at 200 V for approximately 1h. For one OAOA sample a second gel was run in parallel and Sypro Ruby stained, to allow spot picking and mass spectrometry analysis, as described above. Following 1-D and 2-D gel electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad). The resulting membranes were immunoblotted with anti-Vimentin MAb clone V9 (Sigma-Aldrich), followed by anti-mouse HRP-conjugated Ab (Pierce, Rockford, IL, USA) and ECL chemiluminescence (Pierce). Chemiluminescence images were recorded using the VersaDoc-imaging system (Bio-Rad). Densitometric analysis of the images was performed by Quantity One Software v 4.4.0 (Bio-Rad).

## **2.7 Immunofluorescence microscopy**

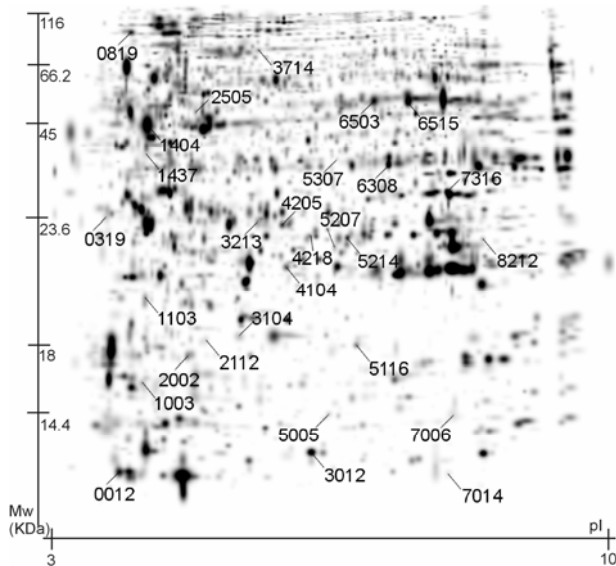
Chondrocytes of cartilage samples of 2 additional healthy patients (NoNo) and 3 additional OA patients were isolated and cultured as described above. In one of the OA patients, no visually intact cartilage was present. Chondrocytes cultivated in alginate beads for 10 days

were washed in PBS for 5 minutes. The medium used during sample processing was a modified Hanks Balanced Salt Solution (mHBSS) as described by Langelier et al. [20]. Chondrocytes were fixed in the alginate beads using 4 % para-formaldehyde (Sigma-Aldrich) for 15 minutes. Fixation was stopped by 3 washes in 100mM glycine. Alginate beads were smeared on poly-l-lysine coated glass slides. Specimens were permeabilized using Triton X-100 (Sigma-Aldrich) 0,5%. Samples were washed and blocked using 5% Normal Goat Serum (NGS) for 1h and were incubated overnight at 4°C with primary mouse anti-vimentin clone V9 (Sigma-Aldrich) in 1% NGS in mHBSS. After three washes, they were incubated with anti-mouse AlexaFluor 488-conjugated secondary antibody (Molecular Probes) in 1% NGS, followed by phalloidin-TRITC (Sigma-Aldrich) staining for 1 h. Samples were washed three times and incubated in DAPI nuclear stain (Molecular Probes). Finally, glass slides were washed four times in mHBSS. The slides were mounted on a coverslip and samples were examined with confocal laser scanning microscopy (Bio-Rad MRC1024). At least 20, randomly selected, individual chondrocytes of each sample were imaged.

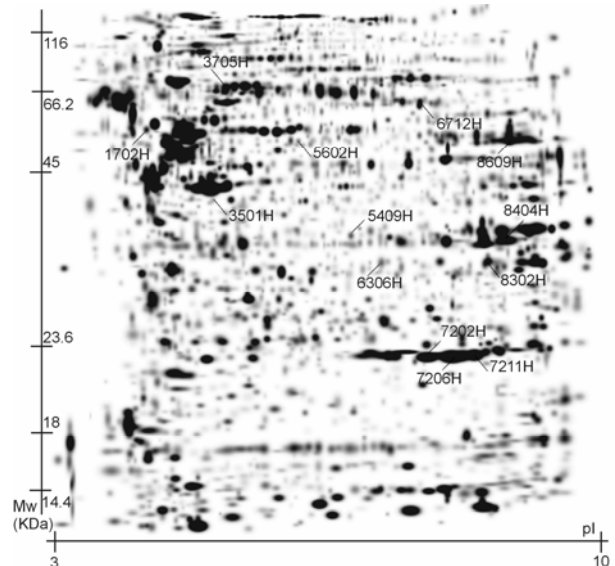
### 3. Results

#### 3.1 *Comparative proteome analysis of normal and OA chondrocytes*

We initiated this study by performing 2-DE, for both the soluble and hydrophobic fractions, on 20 samples (6 healthy (NoNo), 7 visually intact zones of cartilage from OA-patients (NoOA), 7 visually damaged zones of OA-cartilage (OAOA)). Extraction of the proteome from chondrocytes yielded an average cytosolic protein concentration of  $2.39 \pm 0.60 \mu\text{g}/\mu\text{l}$  and an average hydrophobic protein concentration of  $1.94 \pm 0.48 \mu\text{g}/\mu\text{l}$ . Gels were stained, scanned and digitized as described above. The match set's standard images are shown in figure 2 and 3. In the samples of the soluble fraction  $1301 \pm 141$  spots could be detected on each gel. In the hydrophobic fraction  $1212 \pm 96$  spots (Sypro Ruby stain) could be detected. Spots which showed a significant ( $p < 0.05$ ) different intensity between NoNo versus NoOA or OAOA samples in Mann-Whitney U-test were considered as protein spots with a significant altered expression. The identified proteins are shown in table 1 and table 2, respectively. As NoOA and OAOA chondrocytes are isolated from the same knee joint, the corresponding spot intensities were compared using a Wilcoxon paired-sample test. This revealed 17 identified proteins which were differentially ( $p < 0.05$ ) expressed (table 3).



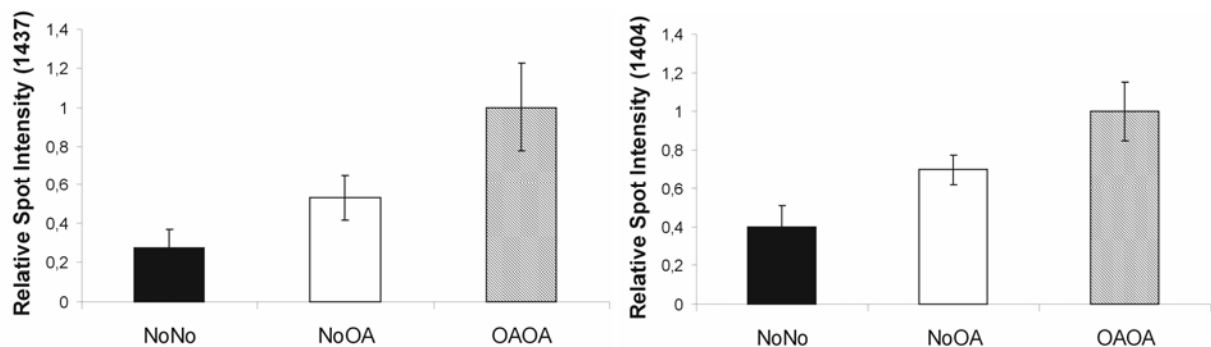
**Figure 2:** Match set's standard image of 2-D separated proteins of the soluble fraction (derived from OAOA sample). Numbered spots are identified by mass spectrometry.



**Figure 3:** Match set's standard image of 2-D separated proteins of the hydrophobic fraction (derived from OAOA sample). Numbered spots are identified by mass spectrometry.

Among the identified spots, several proteins are known to be involved in metabolic pathways (transaldolase, 6-phosphogluconolactonase, alpha-enolase and triose-phosphate isomerase and ATP-synthase ( $\alpha$ -subunit)). Furthermore proteins related to apoptotic pathways (VDAC-proteins and Annexins) and oxidative stress (Peroxisome oxidoreductase 6, Cu/Zn superoxide dismutase, Mn superoxide dismutase) were identified.

Of special interest was the identification of two spots, both upregulated in OAOA, as vimentin (Figure 4). Both spots were located in the gel below the expected molecular weight of native vimentin (Figure 2), suggesting a more pronounced vimentin cleavage in OAOA.



**Figure 4:** Two spots, identified as vimentin, showed a higher expression in OAOA samples. Bars represent relative average expression levels ( $\pm$  SEM) of spots 1404 and 1437 in each group. Black: NoNo, White: NoOA, Shaded: OAOA.



**Table 1:** Identified protein spots, differentially expressed ( $p < 0.05$ ) between NoNo and NoOA samples. The Swiss-Prot accession number is mentioned between brackets. ‘SSP’ indicates the spot number (numbers followed by H refer to spots from hydrophobic fraction). ‘Phosphorylation’ indicates if spot was stained using Pro-Q Diamond. ‘ESI MS/MS’ indicates the number of peptide sequences obtained from ESI Q-TOF used for identification. ‘Ratio’ is the ratio of the average normalized spot intensity of the NoOA- to NoNo-group.

Table 1A: Protein spots showing a higher expression in NoOA vs NoNo				
SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
0819	Glucosidase II beta subunit precursor (P14314)	Yes	5	4.77
2505	Mannose-6-phosphate receptor binding protein 1 (O60664)		4	2.98
3501 H	Stomatin-like protein 2 (Q9UJZ1)		3	2.59
3714	Heat shock protein 70 kDa protein 1 (P08107)		2	2.28
5602 H	Protein disulfide isomerase A3 (P30101)		2	2.00
6308	Annexin A1 (P04083)		5	4.95
6515	Alpha enolase (P06733)		2	1.65
7211 H	Superoxide Dismutase Mn (P04179)		3	2.08
8609 H	ATP-synthase $\alpha$ -chain (P25705)		6	29.37
Table 1B: Protein spots showing a lower expression in NoOA vs NoNo				
SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
1003	Divalent cation tolerant protein CUTA (O60888)		2	0.55
1103	Calcyphosine (Q13938)	Yes	3	0.44
2112	Superoxide Dismutase Cu/Zn (P00441)		1	0.24
4104	DJ-1 protein (Q99497)		2	0.49
4218	Peroxiredoxin 6 (P30041)		6	0.59
7006	Cystatin B (P04080)		2	0.45
7014	Neddylin (Q15843)		2	0.49

**Table 2:** Identified protein spots, differentially expressed ( $p < 0.05$ ) between NoNo and OAOA samples. The Swiss-Prot accession number is mentioned between brackets. ‘SSP’ indicates the spot number (numbers followed by H refer to spots from hydrophobic fraction). ‘Phosphorylation’ indicates if spot was stained using Pro-Q Diamond. ‘ESI MS/MS’ indicates the number of peptide sequences obtained from ESI Q-TOF used for identification. ‘Ratio’ is the ratio of the average normalized spot intensity of the OAOA- to NoNo-group.

Table 2A: Protein spots showing a higher expression in OAOA vs NoNo				
SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
0819	Glucosidase II beta subunit precursor (P14314)	Yes	5	2.47
1404	Vimentin (P08670)		16	2.50
1437	Vimentin (P08670)	Yes	7	3.03
2002	Eukaryotic translation initiation factor 5A (P63241)		1	4.32
3213	Beta-Hexosaminidase beta chain (P07686)		2	3.99
3501 H	Stomatin-like protein 2 (Q9UJZ1)		3	1.81
3705 H	Heat shock cognate 71 kDa protein (P11142)		7	1.62
3714	Heat shock protein 70 kDa protein 1 (P08107)		2	2.52

5602 H	Protein disulfide isomerase A3 (P30101)		2	2.7
6306 H	Voltage-dependent anion-selective channel protein 2 (P45880)		2	1.86
6308	Annexin A1 (P04083)		5	5.23
6503	Alpha enolase (P06733)		3	1.5
6515	Alpha enolase (P06733)		2	1.75
6712 H	Lamin A/C (P02545)		3	2.97
7206 H	Superoxide Dismutase Mn (P04179)	Yes	3	1.76
7211 H	Superoxide Dismutase Mn (P04179)		3	2.91
8212	3-hydroxyacyl-CoA dehydrogenase type II (Q99714)		2	13.83
8302 H	Voltage-dependent anion-selective channel protein 1 (P21796)		3	1.65
8609 H	ATP-synthase $\alpha$ -chain (P25705)		6	52.97

Table 2B: Protein spots showing a lower expression in OAOA vs NoNo

SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
1003	Divalent cation tolerant protein CUTA (O60888)		2	0.65
1103	Calcyphosine (Q13938)	Yes	3	0.51
2112	Superoxide Dismutase Cu/Zn (P00441)		1	0.42
3104	Superoxide Dismutase Cu/Zn (P00441)		2	0.37
4104	DJ-1 protein (Q99497)		2	0.57
4218	Peroxiredoxin 6 (P30041)		6	0.38
5005	Fatty acid-binding protein (P05413)		2	0.33
7006	Cystatin B (P04080)		2	0.55
7014	Neddylin (Q15843)		2	0.55

**Table 3:** Identified protein spots, differentially expressed ( $p < 0.05$ ) between NoOA and OAOA samples. The Swiss-Prot accession number is mentioned between brackets. ‘SSP’ indicates the spot number (numbers followed by H refer to spots from hydrophobic fraction). ‘Phosphorylation’ indicates if spot was stained using Pro-Q Diamond. ‘ESI MS/MS’ indicates the number of peptide sequences obtained from ESI Q-TOF used for identification. ‘Ratio’ is the ratio of the average normalized spot intensity of the OAOA- to NoOA-group.

Table 3A: Protein spots showing a higher expression in OAOA vs NoOA				
SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
3012	S100 calcium-binding protein A4 (P26447)		2	2.11
4205	6-phosphogluconolactonase (O95336)		2	1.84
5116	Cofilin-1 (P23528)	Yes	2	1.45
5207	Protein-L-isoaspartate(D-aspartate) O-methyltransferase (P22061)		3	1.62
5214	Triosephosphate isomerase (P60174)	Yes	3	1.88
5307	Transaldolase (P37837)		2	3.19
5409 H	Annexin A1 (P04083)		3	1.43
6712 H	Lamin A/C (P02545)		3	1.44
7202 H	Superoxide Dismutase Mn (P04179)		2	1.21
7211 H	Superoxide Dismutase Mn (P04179)		3	1.40

7316	Superoxide Dismutase Mn (P04179)	Yes	4	3.15
8212	3-hydroxyacyl-CoA dehydrogenase type II (Q99714)		2	3.27

Table 3B: Protein spots showing a lower expression in OAOA vs NoOA

SSP	Protein	Phosphorylation	ESI MS/MS	Ratio
0012	S-100 calcium-binding protein A1(P23297)	Yes	1	0.68
0319	Acidic leucine-rich nuclear phosphoprotein 32 family member B (Q92688)	Yes	2	0.67
0819	Glucosidase II beta subunit precursor (P14314)	Yes	5	0.52
1702 H	Golgi reassembly stacking protein 2 (Q9H8Y8)		2	0.60
8404 H	Annexin A2 (P07355)		2	0.31

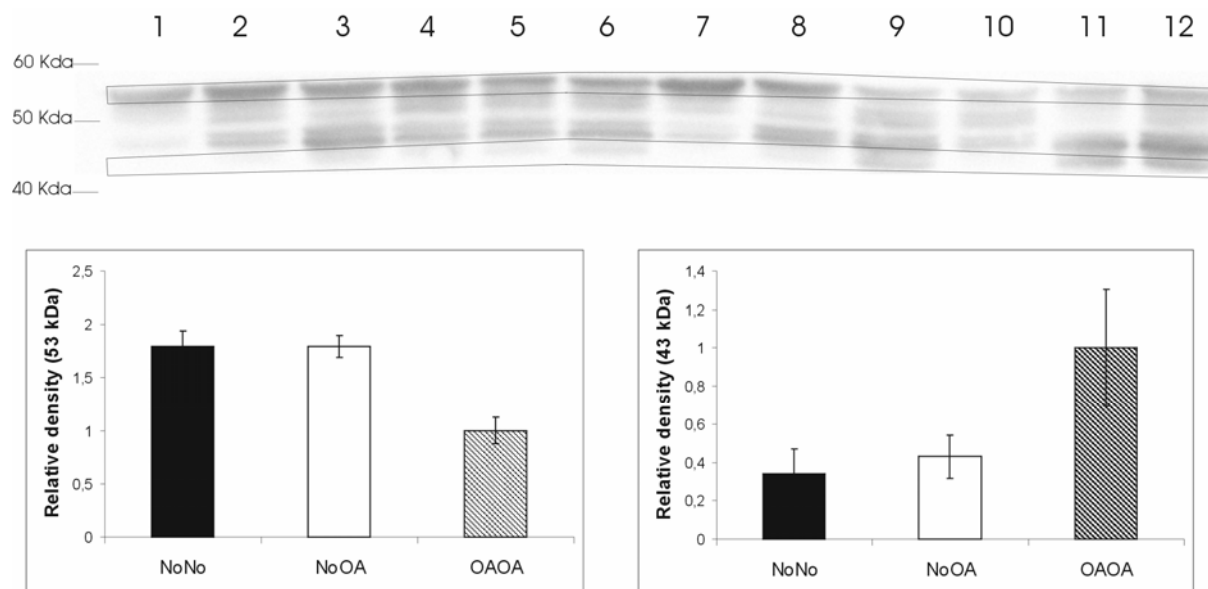
### 3.2 *Several differentially expressed proteins were shown to be phosphorylated, using specific in-gel staining of phosphoproteins*

We next evaluated the phosphorylation status of differentially expressed proteins. Therefore, samples were sequentially stained with Pro-Q Diamond and Sypro Ruby. The Peppermint Stick Phosphoprotein Molecular Weight Standards showed the expected staining pattern for phosphorylated proteins, indicating the selectivity of the Pro-Q Diamond staining procedure (results not shown). In the samples of the soluble fraction  $122 \pm 15$  spots could be detected on each gel, whereas in the hydrophobic fraction  $96 \pm 17$  spots (ProQ-Diamond stain) could be detected. Spots showing a differential expression pattern in the comparative proteome analysis and which stained positive with Pro-Q Diamond are indicated as phosphoproteins in Tables 1, 2 and 3. Among these identified phosphorylated proteins, cofilin-1 is of special interest. Moreover, a known phosphorylation site at Ser-3 could be identified by mass spectrometry (supplementary data, figure 1). Cofilin is a key regulator of actin organisation as this protein is involved in severing and depolymerization of actin filaments (reviewed by DesMarais et al. [21]). As the activity of cofilin is inhibited by phosphorylation [22], the detected upregulation of this protein in OAOA chondrocytes will not necessarily result in a higher activity of this protein. Whether this had a biological impact on actin filaments was further investigated by immunofluorescence (see below).

### 3.3 *Vimentin cleavage was confirmed by western blot analysis*

As the data of the differential proteome analysis points to vimentin cleavage, vimentin and its cleavage products were investigated by 1-D gel electrophoresis and Western blotting, using a monoclonal antibody directed against a C-terminal amino-acid sequence of human vimentin. 1-D Western blots clearly indicate the higher abundance of a band near 53 kDa, the molecular weight of native vimentin, in NoNo and NoOA samples compared to OAOA samples (Figure

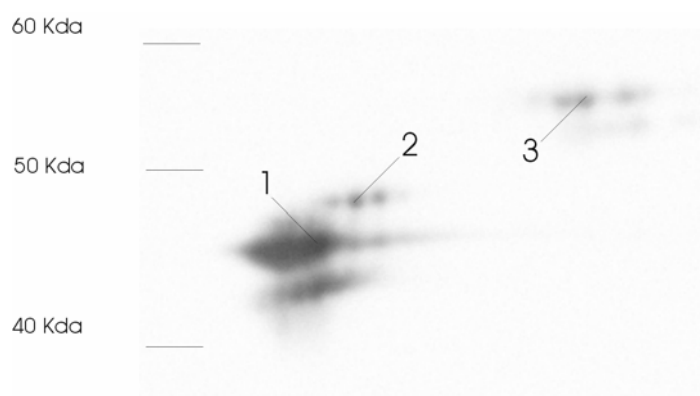
5). Moreover, a lower Mw band (near 43 kDa) of vimentin was found to be more intense in 3 OAOA samples. A less intense staining of vimentin was observed in hydrophobic fractions, which showed less explicit differences between NoNo and NoOA/OAOA samples in the 53 kDa region (supplementary data, figure 2).



**Figure 5:** 1-D Western blot analysis of the soluble fraction of 12 samples (from the left to the right: 4 NoNo samples, 4 NoOA samples and 4 OAOA samples). A: Equal amounts of soluble fractions of chondrocytes were loaded on 10% SDS-PAGE and immunoblotted. Immunoblotting was performed using monoclonal anti-vimentin (clone V9). Where NoNo and NoOA samples show a more intense band at the native molecular weight of vimentin (upper box), 3 OAOA samples show a more intense band near 43 kDa (lower box). B-C: Results of densitometric analysis. Bars represent relative average density ( $\pm$  SEM) of bands per group. Black: NoNo, White: NoOA, Shaded: OAOA.

These findings confirmed the difference between NoNo and OAOA samples observed by 2-DE. In addition, differences between the NoOA and OAOA group were observed. It should be noted however, that in the 2-DE analysis differences in vimentin spot intensities between NoOA and OAOA could not reach statistical significance, but showed p-values of 0,06 and 0,08 for spot 1404 and 1437, respectively. Hence, it may be concluded that both the 1-D and 2-D analysis, point to a higher abundance of low molecular weight vimentin in OAOA compared to NoNo and NoOA samples. To further characterize these bands, a 2-D Western blot was performed of 2 NoNo and 2 OAOA samples. All samples showed the same general trend: a decreased molecular weight between 53 kDa and 43 kDa was accompanied by a shift towards acidic pI (Figure 6). As the N-terminal sequence (78 amino acids) of native vimentin has a high pI (12.01 as computed by the pI/Mw tool at [www.expasy.org](http://www.expasy.org)), cleavage of this sequence will yield a protein with a theoretical pI of 4.71 and Mw of 45 kDa. In contrast,

cleavage of the amino acid sequence at the C-terminus, which yields a protein with a comparable Mw, resulted in a theoretical pI of 5.20. Both the lower pI-values of lower molecular weight fragments and the ability to detect the cleavage products with an antibody directed against a peptide located near the C-terminus, suggest that N-terminus of vimentin is cleaved. This hypothesis was further confirmed by mass spectrometry data. Sequence information about the different vimentin spots was obtained by mass spectrometry analysis of 3 intense spots at different molecular weight (Figure 6). The matched peptides for the different spots are listed in table 4. Mass spectrometry data revealed that none of the matched peptides could be located near the N-terminus for the spots located at 48 kDa or below, while high sequence coverage was achieved for the sequence starting from amino acid 78. Peptides located near the N-terminus could be identified in the higher molecular weight spot. The combination of these data clearly points to N-terminal vimentin cleavage in OAOA.



**Figure 6:** 2-D Western blot of the soluble fraction of OAOA sample. 30 µg of total protein was loaded on 4-7 IPG strip, transferred to 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and vimentin was detected using monoclonal anti-vimentin (clone V9). 1, 2 and 3 indicate the protein spots that were excised from a gel, run in parallel to the gels used for Western blotting.

**Table 4:** Results of the identification of 3 spots, identified as vimentin by Western blotting, at different molecular weight. Spots were excised from a gel run in parallel with the Western blots. Peptides, identified by ESI MS/MS, matched to the theoretical sequence are indicated in bold red. The indicated molecular weights are apparent molecular weights, as estimated by extrapolation of molecular weight markers.

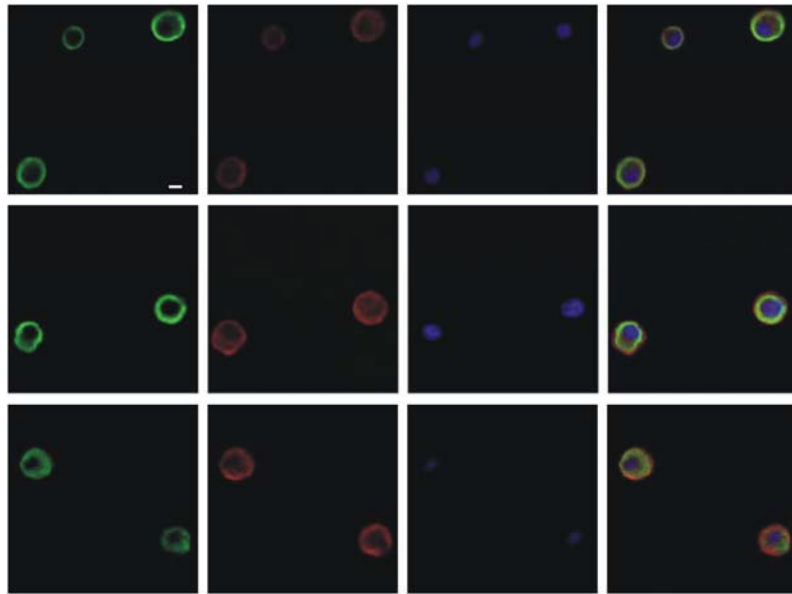
Matched peptides vimentin 3 (Mw: 53 kDa)					
1	STRSVSSSSY	RRMFGGPGTA	SRPSSSRSYV	TTSTR <b>TYSLG</b>	<b>SALRPSTSR</b>
51	<b>LYASSPGGVY</b>	<b>ATRSSAVRLR</b>	SSVPGVRL <b>LQ</b>	<b>DSVDFSLADA</b>	<b>INTEFKNTRT</b>
101	NEK <b>VELQELN</b>	DRFANYIDKV	RFLEQQNK <b>IL</b>	<b>LAELEQLKGQ</b>	<b>GKSRLGDLYE</b>
151	<b>EEMRELRRQV</b>	<b>DQLTNDKARV</b>	EVER <b>DNLAED</b>	<b>IMRLREKLQE</b>	<b>EMLQREEAEN</b>
201	<b>TLQSFRQDVD</b>	<b>NASLARLDLE</b>	<b>RKVESLQEEI</b>	<b>AFLKKLHEEE</b>	<b>IQELQAQIQE</b>
251	QHVQIDVDVS	KPDLTAALRD	VR <b>QQYESVAA</b>	<b>KNLQEAEEWY</b>	<b>KSKFADLSEA</b>
301	<b>ANRNNDALRQ</b>	<b>AKQESTEYRR</b>	<b>QVQSLTCEVD</b>	<b>ALKGTNESLE</b>	<b>RQMREMEENF</b>
351	AVEAANYQDT	IGRL <b>QDEIQN</b>	<b>MKEEMARHLR</b>	<b>EYQDLLNVKM</b>	<b>ALDIEIATYR</b>
401	<b>KLLEGEESRI</b>	<b>SLPLPNFSSL</b>	<b>NLRETNLDSL</b>	<b>PLVDTHSKRT</b>	<b>LLIKTVETRD</b>
451	<b>GQVINETSQH</b>	<b>HDDLE</b>			

Matched peptides vimentin 2 (Mw: 48 kDa)					
1	STRSVSSSSY	RRMFGGPGTA	SRPSSSRYSY	TTSTRTYSLG	SALRPSTSR
51	LYASSPGGVY	ATRSSAVRLR	SSVPGVRLQ	DSVDFSLADA	INTEFKNTRT
101	NEKVELQELN	DRFANYIDKV	RFLEQQNKIL	LAELEQLKGQ	GKSRLGDLYE
151	EEMRELRRQV	DQLTNDKARV	EVERDNLAED	IMRLREKLQE	EMLQREEAEN
201	TLQSFRQDVD	NASLARLDLE	RKVESLQEEI	AFLKKLHEEE	IQELQAQIQE
251	QHVQIDVDVS	KPDLTAALRD	VRQQYESVAA	KNLQEAEEWY	KSKFADLSEA
301	ANRNNDALRQ	AKQESTEYRR	QVQSLTCEVD	ALKGTNESLE	RQMREMEENF
351	AVEAANYQDT	IGRLQDEIQN	MKEEMARHLR	EYQDLLNVKM	ALDIEIATYR
401	KLLEGEESRI	SLPLPNFSSL	NLRETNLDSL	PLVDTHSKRT	LLIKTVETRD
451	GQVINETSQH	HDDLE			
Matched peptides vimentin 1 (Mw: 44 kDa)					
1	STRSVSSSSY	RRMFGGPGTA	SRPSSSRYSY	TTSTRTYSLG	SALRPSTSR
51	LYASSPGGVY	ATRSSAVRLR	SSVPGVRLQ	DSVDFSLADA	INTEFKNTRT
101	NEKVELQELN	DRFANYIDKV	RFLEQQNKIL	LAELEQLKGQ	GKSRLGDLYE
151	EEMRELRRQV	DQLTNDKARV	EVERDNLAED	IMRLREKLQE	EMLQREEAEN
201	TLQSFRQDVD	NASLARLDLE	RKVESLQEEI	AFLKKLHEEE	IQELQAQIQE
251	QHVQIDVDVS	KPDLTAALRD	VRQQYESVAA	KNLQEAEEWY	KSKFADLSEA
301	ANRNNDALRQ	AKQESTEYRR	QVQSLTCEVD	ALKGTNESLE	RQMREMEENF
351	AVEAANYQDT	IGRLQDEIQN	MKEEMARHLR	EYQDLLNVKM	ALDIEIATYR
401	KLLEGEESRI	SLPLPNFSSL	NLRETNLDSL	PLVDTHSKRT	LLIKTVETRD
451	GQVINETSQH	HDDLE			

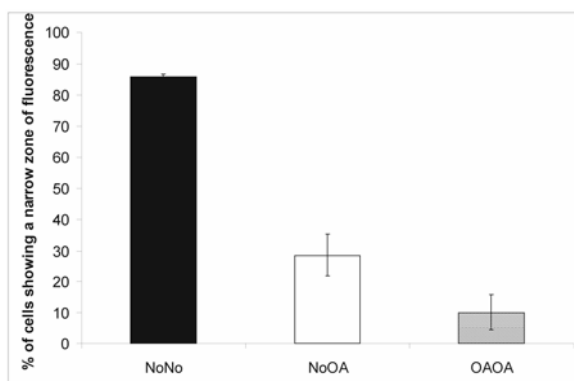
### 3.4 Confocal microscopy of vimentin in chondrocytes points to disrupted vimentin organisation in OA

As it is known that the amino-terminal domain is involved in the intermediate filament assembly [23] and in the interaction of vimentin with the plasma membrane [24], the detected N-terminal cleavage could possibly influence the organisation of vimentin in chondrocytes. Confocal microscopy studies were therefore performed on chondrocyte samples to test this hypothesis. The images show an altered distribution and organisation of vimentin in NoNo chondrocytes compared to OAOA chondrocytes. Representative images of each group are shown in figure 7. While the majority of NoNo chondrocytes clearly show a narrow intense pattern of vimentin expression confined to the cell membrane (figure 8), by contrast OAOA chondrocytes show a more diffuse distribution of fluorescence within the cytoplasm.

As described above phosphorylated cofilin-1, an enzyme involved in actin filament organisation, was differentially expressed. To further elucidate the possible role of this enzyme in chondrocyte biology, actin filaments were stained using phalloidin-TRITC. Analysis of F-actin stained images did not reveal clear differences in F-actin organisation between the sample groups.



**Figure 7:** Representative confocal microscopy images of NoNo (upper panel), NoOA (middle panel) and OAOA chondrocytes (lower panel). Chondrocytes were immunostained for vimentin (green), F-actin (red) and the nucleus (blue), shown as individual channels and in superposition from the left to the right. Scale bar indicates 5  $\mu\text{m}$ . Green panel (vimentin-staining): The majority of NoNo chondrocytes clearly show a narrow zone of fluorescence, in contrast to the spread fluorescence of OAOA chondrocytes. Red panel (F-actin staining): No clear differences in F-actin staining could be detected between the sample groups. All sample groups show a fluorescence signal distributed over the cytoplasm. Blue panel: nuclear DAPI-stain. Right panel: Superimposition of the three images.



**Figure 8:** Percentage of chondrocytes showing a narrow intense zone of fluorescence at the periphery of the cytoplasm (expressed as mean  $\pm$  SD). Cells were incubated with monoclonal anti-vimentin, followed by the appropriate secondary antibody, labelled in Alexa Fluor 488.

#### 4. Discussion

The goal of our study was to identify novel molecular mechanisms or proteins, participating and resulting in cartilage breakdown during the pathogenesis of OA. Therefore, we compared the protein expression patterns of chondrocytes obtained from intact cartilage (NoNo), from the visual intact zones (NoOA) and degenerated zones (OAOA) of OA-cartilage. Chondrocytes were cultured in alginate for 10 days. This culture system allows to selectively isolate the chondrocytes with their cell-associated matrix from the interterritorial matrix. In

alginate hydrogel, chondrocytes maintain their original phenotype and synthesize cartilage-specific matrix components [25].

Interestingly, the NoOA group shows a different expression pattern for a substantial number of proteins in comparison to the NoNo group (see table 1), despite the fact that the chondrocytes are in both cases isolated from visually intact cartilage. These data confirm the hypothesis that chondrocytes undergo phenotypic alterations even before the development of visible cartilage damage [26].

Phenomena such as metabolic activation of OA chondrocytes in an attempt to repair cartilage breakdown [27], oxidative stress [28] and apoptotic processes [29] have previously been reported in the pathogenesis of osteoarthritis. These phenomena are reflected in our analysis by the identification of proteins involved in these biological processes. Proteins involved in pentose-phosphate pathway (transaldolase and 6-phosphogluconolactonase), glycolysis (alpha-enolase and triose-phosphate isomerase) and oxidative phosphorylation (ATP-synthase ( $\alpha$ -subunit)) were found to be upregulated in OA chondrocytes or in OAOA chondrocytes versus NoOA samples. The identification of several proteins involved in these pathways clearly points to a metabolic activation during OA, which is a known phenomenon. Furthermore, an increased role of oxidative phosphorylation in chondrocyte function under conditions of cartilage stress and increased energy demands, as might be associated with OA, has been suggested by Johnson et al [30].

The differential expression of peroxiredoxin 6, an enzyme able to reduce hydroperoxides, Cu/Zn superoxide dismutase (Cu/ZnSOD) and Mn superoxide dismutase (MnSOD) illustrates the involvement of oxidative stress in the pathogenesis of OA. The biological role of Cu/ZnSOD and MnSOD, was previously reported in chondrocytes. In contrast the identification of peroxiredoxin 6, as a novel differentially expressed protein in OA, further contributes to the elucidation of oxidative stress pathways in the pathogenesis of OA.

The role of apoptosis in OA is reflected by the identification of voltage-dependent anion channels (VDAC-1 and VDAC-2) and Annexin A1, which are proteins known to be involved in apoptotic processes [31] [32] [33]. The identification of VDAC-proteins, which have never been described in chondrocytes, can shed a new light on apoptotic processes in chondrocytes.

The cytoskeletal architecture, which consists of a network of actin microfilaments, microtubules and intermediate filaments (IFs), plays a significant role in the physiology of



chondrocytes. Furthermore, the cytoskeleton is important in biomechanics and is involved in mechanotransduction signalling pathways [34]. Vimentin IFs have been implicated in the mechanical integration of the cellular space [35]. In chondrocytes it has been suggested that the vimentin cytoskeleton could be implicated in mechanotransduction [20]. 2-D gel electrophoresis, mass spectrometry and Western blot data reveal a more pronounced N-terminal cleavage of vimentin in OA. Since the amino-terminal domain of vimentin is involved in IF assembly [23] and in interaction of vimentin with the plasma membrane [24], we hypothesized that this vimentin cleavage would be reflected in an altered vimentin organisation in OA chondrocytes compared to healthy chondrocytes. This is confirmed by immunofluorescent labelling of vimentin in chondrocytes: the more diffuse distribution of the fluorescent signal in OAOA chondrocytes over the cytoplasm, in contrast to intense fluorescence at the periphery in NoNo chondrocytes, probably reflects a disturbance in vimentin IF organisation and/or a diminished interaction of vimentin with the plasma membrane. Taken together, the combination of spot analysis data, 1-D and 2-D Western blotting, high sequence coverage mass spectrometry data and confocal microscopy images points indisputably to a disturbed vimentin organisation in OAOA chondrocytes.

Trickey et al [36] demonstrated the possible involvement of vimentin IFs in the visco-elastic properties of human articular chondrocytes. Moreover, it has recently been shown that disassembly of the vimentin cytoskeleton, induced by acrylamide, disrupts chondrocyte homeostasis [37]. In consequence, the observed vimentin distortion probably plays a role in the pathogenesis of OA.

Vimentin cleavage is a process which is described during apoptosis [38, 39]. Caspase-3 and caspase-8 are reported to be the proteases involved in apoptosis-related vimentin cleavage [40]. As a higher caspase-3 expression in OA-chondrocytes is documented [29], this enzyme is a candidate mediator for vimentin cleavage in OA. Nevertheless, the fragments, representing the most intense spots near 44 and 48 kDa, were investigated by mass spectrometry and contained the known cleavage site of caspase-3 and caspase-7 (AA 85) and caspase-6 and caspase-8 (AA 259) [40, 41]. This observation makes caspase-3 cleavage very unlikely. In addition, vimentin cleavage seems to be a phenomenon taking place in the basal metabolism of chondrocytes, since cleavage products were also detected in healthy samples. These findings allow us to suggest that caspase-mediated apoptotic processes are probably not involved in the observed vimentin cleavage.

The primary cause of vimentin distortions remains unclear. It could be hypothesized that excessive mechanical load will induce an altered vimentin organisation, making vimentin

more susceptible to proteases. This hypothesis is supported by the suggestion of Durrant et al. [42] that chondrocytes modify their vimentin cytoskeleton in response to changing mechanical conditions. Alternatively, the observed vimentin alterations could be induced by imbalances in the autocrine and paracrine anabolic and catabolic pathways associated with OA. In OA chondrocytes the catabolic pathways override the anabolic pathways [26], which may result in OA-associated vimentin breakdown, possibly via downstream regulation of proteases or chaperone proteins.

In conclusion, this study confirmed the metabolic activation of chondrocytes isolated from OA-cartilage and clearly demonstrated vimentin cytoskeleton disruption in OA-affected chondrocytes. These observations may shed new light on the pathogenesis of OA. Whether primary processes, involved in vimentin cleavage and cytoskeleton breakdown, are based on mechanical phenomena, on metabolic imbalances or on a combination of both will need further research.

## Acknowledgements

The authors acknowledge Dr. B. Lucas for assistance by the acquisition of confocal microscopy images and S. Vandecasteele for the technical assistance in mass spectrometry.

## 5. References

- [1] Huang, W., Li, W. Q., Dehnade, F., Zafarullah, M., Tissue inhibitor of metalloproteinases-4 (TIMP-4) gene expression is increased in human osteoarthritic femoral head cartilage. *J Cell Biochem* 2002, 85, 295-303.
- [2] Aigner, T., Vornehm, S. I., Zeiler, G., Dudhia, J., *et al.*, Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. *Arthritis Rheum* 1997, 40, 562-569.
- [3] Duerr, S., Stremme, S., Soeder, S., Bau, B., Aigner, T., MMP-2/gelatinase A is a gene product of human adult articular chondrocytes and is increased in osteoarthritic cartilage. *Clin Exp Rheumatol* 2004, 22, 603-608.
- [4] Wang, M. X., Wei, A., Yuan, J., Trickett, A., *et al.*, Expression and regulation of peroxiredoxin 5 in human osteoarthritis. *FEBS Lett* 2002, 531, 359-362.
- [5] Wang, J., Elewaut, D., Veys, E. M., Verbruggen, G., Insulin-like growth factor 1-induced interleukin-1 receptor II overrides the activity of interleukin-1 and controls the homeostasis of the extracellular matrix of cartilage. *Arthritis Rheum* 2003, 48, 1281-1291.
- [6] Aigner, T., Zien, A., Gehrsitz, A., Gebhard, P. M., McKenna, L., Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. *Arthritis Rheum* 2001, 44, 2777-2789.
- [7] De Ceuninck, F., Dassencourt, L., Anract, P., The inflammatory side of human chondrocytes unveiled by antibody microarrays. *Biochem Biophys Res Commun* 2004, 323, 960-969.
- [8] Aigner, T., Fundel, K., Saas, J., Gebhard, P. M., *et al.*, Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. *Arthritis Rheum* 2006, 54, 3533-3544.
- [9] Ruiz-Romero, C., Lopez-Armada, M. J., Blanco, F. J., Proteomic characterization of human normal articular chondrocytes: a novel tool for the study of osteoarthritis and other rheumatic diseases. *Proteomics* 2005, 5, 3048-3059.
- [10] Garcia, B. A., Platt, M. D., Born, T. L., Shabanowitz, J., *et al.*, Protein profile of osteoarthritic human articular cartilage using tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2006, 20, 2999-3006.
- [11] Hermansson, M., Sawaji, Y., Bolton, M., Alexander, S., *et al.*, Proteomic analysis of articular cartilage shows increased type II collagen synthesis in osteoarthritis and expression of inhibin betaA (activin A), a regulatory molecule for chondrocytes. *J Biol Chem* 2004, 279, 43514-43521.
- [12] Aigner, T., Bartnik, E., Sohler, F., Zimmer, R., Functional genomics of osteoarthritis: on the way to evaluate disease hypotheses. *Clin Orthop Relat Res* 2004, S138-143.
- [13] Meachim, G., Light microscopy of Indian ink preparations of fibrillated cartilage. *Ann Rheum Dis* 1972, 31, 457-464.

- [14] Verbruggen, G., Wang, J., Wang, L., Elewaut, D., Veys, E. M., *Analysis of chondrocyte functional markers and pericellular matrix components by flow cytometry*, Humana Press, Totowa, New Jersey 2004.
- [15] Guo, J. F., Jourdian, G. W., MacCallum, D. K., Culture and growth characteristics of chondrocytes encapsulated in alginate beads. *Connect Tissue Res* 1989, 19, 277-297.
- [16] Verbruggen, G., Veys, E. M., Wieme, N., Malfait, A. M., *et al.*, The synthesis and immobilisation of cartilage-specific proteoglycan by human chondrocytes in different concentrations of agarose. *Clin Exp Rheumatol* 1990, 8, 371-378.
- [17] Hauselmann, H. J., Aydelotte, M. B., Schumacher, B. L., Kuettner, K. E., *et al.*, Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. *Matrix* 1992, 12, 116-129.
- [18] Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., *et al.*, Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis* 1998, 19, 837-844.
- [19] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., *et al.*, Chronically inflamed synovium from spondyloarthropathy and rheumatoid arthritis investigated by protein expression profiling followed by tandem mass spectrometry. *Proteomics* 2005, 5, 2247-2257.
- [20] Langelier, E., Suetterlin, R., Hoemann, C. D., Aebi, U., Buschmann, M. D., The chondrocyte cytoskeleton in mature articular cartilage: structure and distribution of actin, tubulin, and vimentin filaments. *J Histochem Cytochem* 2000, 48, 1307-1320.
- [21] DesMarais, V., Ghosh, M., Eddy, R., Condeelis, J., Cofilin takes the lead. *J Cell Sci* 2005, 118, 19-26.
- [22] Moriyama, K., Iida, K., Yahara, I., Phosphorylation of Ser-3 of cofilin regulates its essential function on actin. *Genes Cells* 1996, 1, 73-86.
- [23] Beuttenmuller, M., Chen, M., Janetzko, A., Kuhn, S., Traub, P., Structural elements of the amino-terminal head domain of vimentin essential for intermediate filament formation in vivo and in vitro. *Exp Cell Res* 1994, 213, 128-142.
- [24] Georgatos, S. D., Weaver, D. C., Marchesi, V. T., Site specificity in vimentin-membrane interactions: intermediate filament subunits associate with the plasma membrane via their head domains. *J Cell Biol* 1985, 100, 1962-1967.
- [25] Almqvist, K. F., Wang, L., Wang, J., Baeten, D., *et al.*, Culture of chondrocytes in alginate surrounded by fibrin gel: characteristics of the cells over a period of eight weeks. *Ann Rheum Dis* 2001, 60, 781-790.
- [26] Wang, J., Verdonk, P., Elewaut, D., Veys, E. M., Verbruggen, G., Homeostasis of the extracellular matrix of normal and osteoarthritic human articular cartilage chondrocytes in vitro. *Osteoarthritis Cartilage* 2003, 11, 801-809.
- [27] Hamerman, D., The biology of osteoarthritis. *N Engl J Med* 1989, 320, 1322-1330.

- [28] Henrotin, Y. E., Bruckner, P., Pujol, J. P., The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage* 2003, *11*, 747-755.
- [29] Sharif, M., Whitehouse, A., Sharman, P., Perry, M., Adams, M., Increased apoptosis in human osteoarthritic cartilage corresponds to reduced cell density and expression of caspase-3. *Arthritis Rheum* 2004, *50*, 507-515.
- [30] Johnson, K., Jung, A., Murphy, A., Andreyev, A., *et al.*, Mitochondrial oxidative phosphorylation is a downstream regulator of nitric oxide effects on chondrocyte matrix synthesis and mineralization. *Arthritis Rheum* 2000, *43*, 1560-1570.
- [31] Rapizzi, E., Pinton, P., Szabadkai, G., Wieckowski, M. R., *et al.*, Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca<sup>2+</sup> microdomains to mitochondria. *J Cell Biol* 2002, *159*, 613-624.
- [32] Solito, E., de Coupade, C., Canaider, S., Goulding, N. J., Perretti, M., Transfection of annexin 1 in monocytic cells produces a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation. *Br J Pharmacol* 2001, *133*, 217-228.
- [33] Debret, R., El Btaouri, H., Duca, L., Rahman, I., *et al.*, Annexin A1 processing is associated with caspase-dependent apoptosis in BZR cells. *FEBS Lett* 2003, *546*, 195-202.
- [34] Wang, N., Butler, J. P., Ingber, D. E., Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 1993, *260*, 1124-1127.
- [35] Lazarides, E., Intermediate filaments as mechanical integrators of cellular space. *Nature* 1980, *283*, 249-256.
- [36] Trickey, W. R., Vail, T. P., Guilak, F., The role of the cytoskeleton in the viscoelastic properties of human articular chondrocytes. *J Orthop Res* 2004, *22*, 131-139.
- [37] Blain, E. J., Gilbert, S. J., Hayes, A. J., Duance, V. C., Disassembly of the vimentin cytoskeleton disrupts articular cartilage chondrocyte homeostasis. *Matrix Biol* 2006, *25*, 398-408.
- [38] Prasad, S. C., Thraves, P. J., Kuettel, M. R., Srinivasarao, G. Y., *et al.*, Apoptosis-associated proteolysis of vimentin in human prostate epithelial tumor cells. *Biochem Biophys Res Commun* 1998, *249*, 332-338.
- [39] Hashimoto, M., Inoue, S., Ogawa, S., Conrad, C., *et al.*, Rapid fragmentation of vimentin in human skin fibroblasts exposed to tamoxifen: a possible involvement of caspase-3. *Biochem Biophys Res Commun* 1998, *247*, 401-406.
- [40] Morishima, N., Changes in nuclear morphology during apoptosis correlate with vimentin cleavage by different caspases located either upstream or downstream of Bcl-2 action. *Genes Cells* 1999, *4*, 401-414.
- [41] Byun, Y., Chen, F., Chang, R., Trivedi, M., *et al.*, Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. *Cell Death Differ* 2001, *8*, 443-450.

[42] Durrant, L. A., Archer, C. W., Benjamin, M., Ralphs, J. R., Organisation of the chondrocyte cytoskeleton and its response to changing mechanical conditions in organ culture. *J Anat* 1999, *194* (Pt 3), 343-353.

## **Chapter III: Results**

*Part 2:  $\alpha$ Bcrystallin, a potential mediator of matrix gene expression in chondrocytes during the development of osteoarthritis*





**Regulation of matrix gene expression by the small heat-shock protein,  
 $\alpha$ Bcrystallin, during the development of osteoarthritis**

Stijn Lambrecht<sup>1</sup>, PharmD, Gust Verbruggen<sup>2</sup>, MD, PhD, Dirk Elewaut<sup>2</sup>, MD, PhD, Dieter Deforce<sup>1</sup>, PharmD, PhD

<sup>1</sup>Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium; <sup>2</sup>Department of Rheumatology, University Hospital Ghent, B-9000 Ghent, Belgium

**Corresponding author:** Prof. Dieter Deforce; Laboratory of Pharmaceutical Biotechnology, Harelbekestraat 72, B-9000 Ghent, Belgium; e-mail: [dieter.deforce@ugent.be](mailto:dieter.deforce@ugent.be); Fax: +32-9-220-6688

S. Lambrecht is supported by a grant of the Fund for Scientific Research-Flanders (Belgium).

**To be submitted for publication**

**UK Provisional Patent GB 07/205024**

## Abstract

*Objective:*  $\alpha$ Bcrystallin belongs to the family of small heat-shock proteins. This family of proteins is only poorly described in chondrocytes. We aimed to investigate the expression levels of  $\alpha$ Bcrystallin in chondrocytes isolated from healthy and osteoarthritis (OA)-affected patients. The functional role of this potentially interesting protein in chondrocyte metabolism was further explored using RNA-interference.

*Methods:* Western blot and real-time RT-PCR analysis were performed to confirm the differential expression of  $\alpha$ Bcrystallin between normal and OA chondrocytes cultured in alginate beads. Chondrocytes were stimulated with IL-1 $\beta$  and TNF- $\alpha$  to assess the influence of these cytokines on  $\alpha$ Bcrystallin protein expression. RNA-interference mediated gene knock-down was used to explore the role of this small heat shock protein in chondrocyte biology by transfecting low concentrations of siRNA in cultured chondrocytes.

*Results:* We initially identified  $\alpha$ Bcrystallin as a differentially expressed small heat-shock protein between healthy and OA-affected chondrocytes. The decreased abundance of this protein in OA chondrocytes was confirmed by Western blot. Moreover, real-time RT-PCR confirmed the differential expression between chondrocytes isolated from visually intact and visually damaged zones of OA cartilage. The pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , both down regulated  $\alpha$ Bcrystallin expression. Transfection of low concentrations siRNA in cultured chondrocytes resulted in an efficient knock down of  $\alpha$ Bcrystallin gene expression. This was accompanied by an altered expression of the chondrocyte specific genes BMP-2, aggrecan and collagen type II.

*Conclusion:* This report identified the small heat shock protein  $\alpha$ Bcrystallin as a novel mediator of chondrocyte matrix gene expression that may contribute to the altered chondrocyte metabolism during the development of OA.

## 1. Introduction

$\alpha$ Bcrystallin (HSPb5) belongs to the family of small heat shock proteins (sHSPs), which are a family of proteins with molecular weights below 30 kDa [1], whose function is to protect cells against stress factors. The expression of sHSPs may be induced by various stresses, such as heat-shock, oxidative stress and chemical stresses. These molecular chaperones have been implicated in many different cellular processes (reviewed in [2]). As chaperone proteins, sHSPs interact with several cytoskeletal elements. It has been shown that  $\alpha$ Bcrystallin interacts with the intermediate filaments during mitosis [3] and that intermediate filament actions can be altered by  $\alpha$ Bcrystallin [4]. In addition, this small heat-shock protein is involved in modulating the actin filament dynamics [5]. Kamradt et al described  $\alpha$ Bcrystallin as an inhibitor of TRAIL-induced apoptosis [6] and described it as a negative regulator of caspase-3 activation [7]. The expression of  $\alpha$ Bcrystallin decreased the level of reactive oxygen species (ROS) and abolished the burst of intracellular ROS induced by TNF $\alpha$  [8]. Furthermore,  $\alpha$ Bcrystallin has been related to cellular differentiation in several reports. Ito et al [9], described increasing levels of  $\alpha$ Bcrystallin during differentiation of C2C12 cells. Moreover, it has been shown that  $\alpha$ Bcrystallin expression was induced during the early stages of differentiation of the chondroprogenitor cell line ATDC5 [10]. Another small heat shock protein, HSP 25 (termed HSP 27 in human cells), has been shown to interfere with the differentiation of the C1 cell-line [11].

Based on its ability to interfere with diverse cellular events, it is not surprising that mutations in small heat shock protein genes are implicated in different diseases. For example, mutations in the  $\alpha$ Bcrystallin gene are associated with the neuromuscular disorder desmin-related myopathy [12] or with cataract [13]. Furthermore, this family of proteins have been related with cancer, Alzheimer, Parkinson and other neurodegenerative diseases (reviewed in [14]).

Chondrocytes, the only resident cells in human articular cartilage, are generally considered to be key players in the cartilage degeneration associated with OA [15, 16]. The chondrocyte's metabolism is influenced by a variety of cytokines and growth factors. Some are considered to be anabolic factors (IGF-I, TGF- $\beta$ , BMPs), others are catabolic (IL-1, TNF- $\alpha$ ). The balance between these anabolic and catabolic factors is of major importance to maintain a normal homeostasis in the chondrocyte's metabolism. Until now, the knowledge of the processes involved in the pathogenesis of OA at the molecular level is limited. The disclosure of differentially expressed proteins and their subsequent study might help to better understand

events associated with the development of OA. This knowledge is crucial for the rational development of disease-modifying drugs.

At present, little is known about small heat shock proteins in chondrocytes and articular cartilage and their potential role in the chondrocyte's biology. Based on a differential proteome analysis [17], we compared the expression of  $\alpha$ Bcrystallin in samples of chondrocytes isolated from healthy and OA-cartilage. Furthermore, we investigated the effect of pro-inflammatory cytokines on the expression levels of this protein. The functional role of  $\alpha$ Bcrystallin in the chondrocyte's biology was explored using RNA interference (RNAi): Knock down of the  $\alpha$ Bcrystallin gene expression by siRNA, revealed an altered expression of cartilage matrix genes. .

## 2 Materials and Methods

### 2.1 2-DE analysis

In a proteomics screening study, protein extracts of chondrocytes (6 NoNo, 7 NoOA and 7 OAOA samples) were analyzed by a 2-DE approach, as described in [17]. Briefly, soluble and hydrophobic fractions of protein extracts of chondrocytes were separated by 2-DE. Sypro Ruby stained gels were scanned and analyzed using PDQuest V 7.1. Statistically significant differentially expressed spots ( $p < 0.05$ ; Mann-Whitney U-test) were excised and subjected to tandem mass spectrometry for identification.

### 2.2 Isolation of chondrocytes

Human articular chondrocytes were isolated as previously described. Articular knee cartilage from donors without arthropathy (NoNo) (3 male, 2 female, mean age:  $44 \pm 25$  years) was obtained within 24 h post-mortem. All donors had died as a result of trauma or a brief illness and none of them had been receiving corticosteroids or cytostatic drugs. OA affected cartilage was obtained from patients (6 male, 11 female, mean ages:  $62 \pm 11$  years) within 24 h from total knee arthroplasty. The cartilage from each of these patients was separated in visually intact cartilage (NoOA) and cartilage showing OA-lesions (OAOA). This study was approved by the local Ethics Committee. The cartilage obtained was diced into small fragments and chondrocytes were isolated by sequential enzymatic digestion (hyaluronidase, pronase, collagenase (Sigma-Aldrich, Steinheim, Germany)) as described in detail elsewhere [18]. Trypan blue exclusion revealed that  $>95\%$  of the cells were viable after isolation.

### **2.3    *Culture of chondrocytes in alginate gel***

Chondrocyte cultures in alginate beads were prepared as described by Guo et al [19], with some modifications [20]. The beads were maintained in a 6-well plate (20 beads/well;  $\pm$  50.000 chondrocytes/bead) containing DMEM (Gibco) with 10% fetal calf serum, antibiotics and antimycotics (Gibco) in an incubator at 37°C and in 5% CO<sub>2</sub>. Medium was replaced three times a week for 10 days. After the culture period, the medium was aspirated and the alginate beads were washed and dissolved by incubation in 55 mM tri-sodium citrate dihydrate pH 6.8, at room temperature. The resulting suspension was centrifuged at 1500 rpm for 10 min to separate cells with their CAM from the constituents of the interterritorial matrix. The resulting cell-pellet was washed three times with PBS.

### **2.4    *Western Blot analysis***

Cell lysates of chondrocytes were prepared by resuspending cell pellets in 40mM Tris from the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA), containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and a phosphatase inhibitor-cocktail (Sigma-Aldrich, Steinheim, Germany). Cells were lysed by sonication and the soluble proteins were isolated by centrifugation. Equal amounts (30 µg as determined by 2-D Quant kit, GE Healthcare, Fairfield, USA) of soluble fractions of 13 samples (5 NoNo, 4 NoOA and 4 OAOA) were loaded on 10% SDS-PAGE gel. Equal loading was verified by Ponceau S staining (data not shown). MagicMark (Invitrogen, Paisley, UK) protein standards were run as molecular weight markers. Following 1-D gel electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad). The resulting membranes were immunostained with rabbit anti- $\alpha$ Bcrystallin (FL-175) (Santa-Cruz, Biotechnology, Santa Cruz, USA) followed by an anti-rabbit HRP-conjugated secondary antibody (Pierce, Rockford, IL, USA) and ECL chemiluminescence detection (Supersignal West Dura Extended Duration Substrate, Pierce). Chemiluminescence images were recorded using the VersaDoc-imaging system (Bio-Rad). Image analysis was performed by Quantity One v 4.4.0 (Bio-Rad).

### **2.5    *Immunofluorescence microscopy***

Chondrocytes, cultured in alginate beads, were washed in PBS for 5 minutes. The medium used during sample processing was a modified Hanks Balanced Salt Solution (mHBSS). Chondrocytes were fixed in the alginate beads using 4 % para-formaldehyde (Sigma-Aldrich) for 15 minutes. Fixation was stopped by 3 washes in 100mM glycine. Alginate beads were

smeared on poly-l-lysine coated glass slides. Specimens were permeabilized using Triton X-100 (Sigma-Aldrich) 0,5%. Samples were washed and blocked using 5% Normal Goat Serum (NGS) for 1h and were incubated overnight at 4°C with primary mouse monoclonal IgG<sub>1</sub> anti- $\alpha$ Bcrystallin (Abcam, Cambridge, UK) in 1% NGS in mHBSS. After three washes, they were incubated with anti-mouse IgG<sub>1</sub> AlexaFluor 488-conjugated secondary antibody (Molecular Probes) in 1% NGS for 1 h. Isotype controls were prepared and analyzed in parallel as negative controls. Samples were washed three times and incubated in DAPI nuclear stain (Molecular Probes). Finally, glass slides were washed four times in mHBSS. The slides were mounted on a coverslip and samples were examined with an AxioVert fluorescence microscope equipped with an ApoTome-module (Carl Zeiss). This allows to visualize cells in a near confocal-like way. At least 20, randomly selected, individual chondrocytes of the samples were imaged.

## **2.6 Chondrocyte culture with pro-inflammatory cytokines**

Chondrocytes were isolated from 4 additional patients (2 male, 2 female) and cultured as described above for 7 days with IL-1 $\beta$  (0,1 ng/ml; 0,5 ng/ml) or TNF- $\alpha$  (10 ng/ml). Each stimulation was performed in duplicate per patient. After the culture period cells were isolated, proteins extracted, separated and blotted as described above.

## **2.7 Real-time PCR**

Chondrocytes from 6 patients (1 male, 5 female) were isolated and cultured as described above. After the culture period, Trizol (Invitrogen) was added to the isolated cells, and RNA was extracted according to the manufacturer's instructions, followed by an additional purification step (RNeasy mini-kit (Qiagen)). This step included the digestion of DNA by deoxyribonuclease I (Invitrogen). cDNA was synthesized with oligo(dT) primers using the Superscript kit (Invitrogen).

Real-time PCR was performed using the ABI 7000 Sequence Detection System (Applied Biosystems). Each reaction utilized 20  $\mu$ l of iTaq Supermix with Rox (Bio-Rad, Hercules, CA) and 5  $\mu$ l of cDNA and was performed in triplicate. The thermocycler conditions were 2 min at 50°C, followed by 2 min at 95°C and 45 cycles, each at 95°C for 15 s and 60°C for 1 min. Expression levels were normalized to those of human GAPDH, HPRT and PPIA. Relative quantization was calculated using the  $2^{-\Delta\Delta C_t}$  method [21, 22].

## 2.8 RNAi

Chondrocytes, isolated from visually intact cartilage of OA-patients, were cultured in alginate as described above. Experiments were performed on 4 different patient samples. After the culture period, cells were isolated and seeded in monolayer culture at a density of 50.000 cells/cm<sup>2</sup>. Cells were transfected with 5nM siRNA (final concentration) directed against the  $\alpha$ Bcrystallin mRNA (Qiagen, Venlo, The Netherlands) or with a non-silencing control (All Stars Negative control siRNA, Qiagen), using HiPerfect transfection reagent (Qiagen). Separate transfections using siRNA directed against different sequences in the  $\alpha$ Bcrystallin mRNA strand have been performed. The transcript sequences targeted by the different siRNA sequences were: 5'- CAGGTTCTCTGTCAACCTGGA-3' and 5'- CTCCAGGGAGTTCCACAGGAA-3'. Cells were harvested at 48 hours and cDNA was synthesized as described above. Cell viability after transfection was at least 95% as determined by trypan blue exclusion.

## 3 Results

### ***3.1 Differential proteome analysis of healthy and OA chondrocytes shows the differential expression of a protein identified as $\alpha$ Bcrystallin***

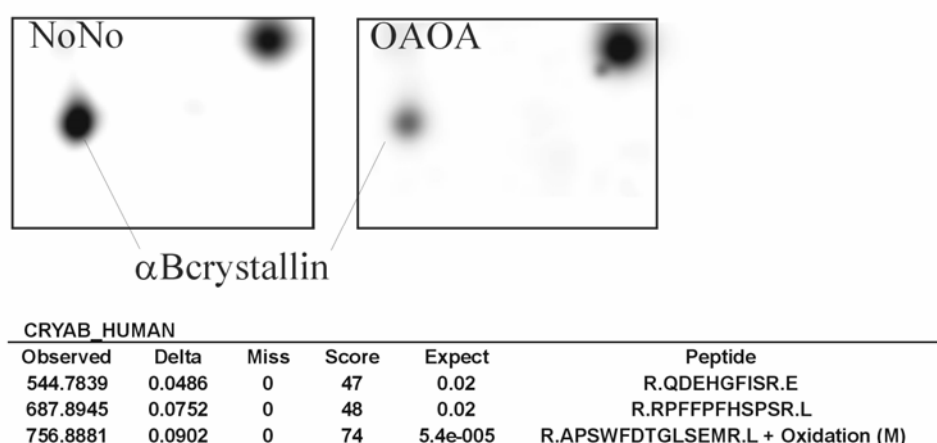
In a previous study, a two-dimensional gel-based differential proteome analysis was performed [17]. Protein expression patterns of chondrocytes isolated from 7 healthy patients and 6 OA-patients were compared. This revealed the differential expression between normal and OA chondrocytes of a spot, which has now been identified as  $\alpha$ Bcrystallin (figure 1). This spot showed a down-regulation in OA samples (0,46 fold,  $p = 0,015$ ) compared to healthy chondrocytes.

### ***3.2 Western blot analysis confirms differential abundance of $\alpha$ Bcrystallin, a protein localized in the cytoplasm***

Expression levels of  $\alpha$ Bcrystallin were compared in lysates of chondrocytes isolated from healthy and OA-cartilage. Proteins extracted from chondrocytes isolated from healthy cartilage (NoNo, 5 patients) and visually intact NoOA and visually damaged (OAOA) OA-cartilage (4 patients) were separated by 1-D gelelectrophoresis and electroblotted. Western blot analysis confirmed the higher abundance of  $\alpha$ Bcrystallin in NoNo samples. There was a gradual decrease in the abundance of  $\alpha$ Bcrystallin from NoNo, over NoOA, to OAOA samples. In addition, each individual OA-patient showed a higher intensity for  $\alpha$ Bcrystallin in

NoOA versus OAOA chondrocytes. The results of the densitometric analysis are shown in figure 2 (panel A and B). Western blots have been performed on albumin depleted synovial fluids of 3 OA, 3 RA and 3 SpA patients (up to 100  $\mu$ g of protein was loaded on gel) and  $\alpha$ Bcrystallin could not be detected in these samples (results not shown).

To determine the subcellular localization of  $\alpha$ Bcrystallin in chondrocytes, we performed immunofluorescence experiments. As shown in Figure 2C,  $\alpha$ Bcrystallin is prominently expressed in the cytoplasm.

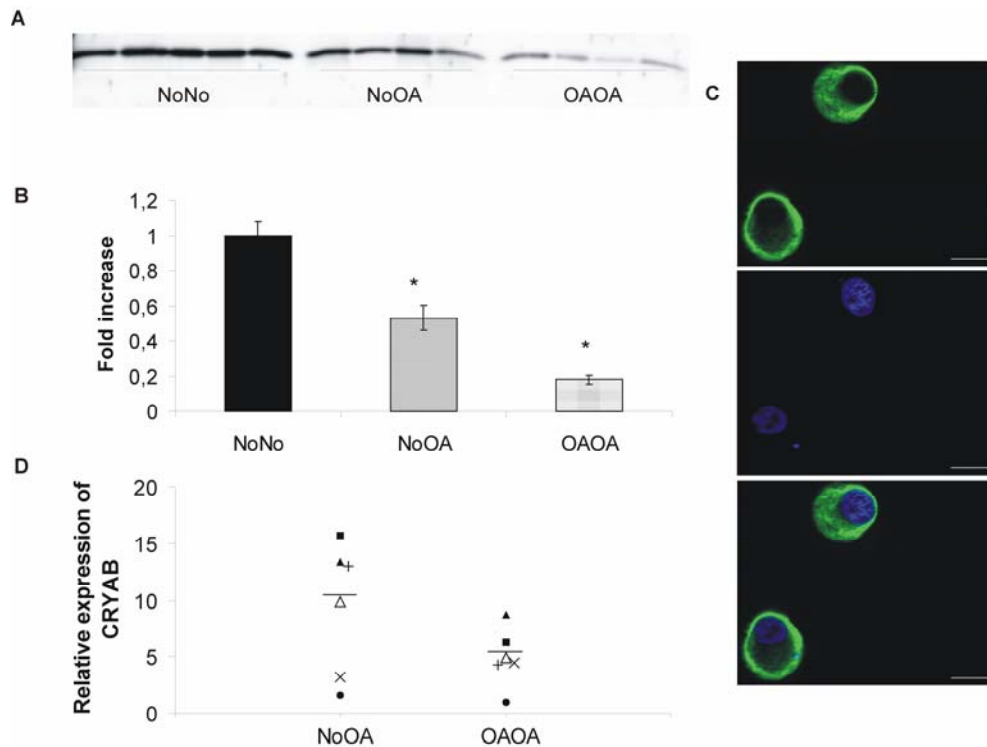


**Figure 1:** Representative zoom-image of the 2-DE gels of chondrocytes isolated from healthy cartilage (NoNo) and chondrocytes isolated from visually damaged zones of OA-cartilage (OAOA). The spot was reliably identified as  $\alpha$ Bcrystallin by tandem mass spectrometry: Observed: Indicates the observed m/z. Delta: Indicates the mass difference (Da) between the observed and the theoretical precursor ion mass; Miss: indicates the number of missed cleavage sites in a peptide; Score: Indicates the Mascot Ions Score; Expect: Indicates the expectation value for the peptide match; Peptide: indicates the identified peptide sequence in a 1-letter amino acid code.

### 3.3 Real-time RT-PCR confirms the differential transcription of $\alpha$ Bcrystallin at the mRNA level

To evaluate whether the differential abundance of  $\alpha$ Bcrystallin is a consequence of a differential gene expression, mRNA levels for  $\alpha$ Bcrystallin were compared by real-time RT-PCR in 6 additional paired samples of NoOA and OAOA chondrocytes. Consistent with the results at the protein level, the mRNA levels of  $\alpha$ Bcrystallin were upregulated in NoOA chondrocytes compared to OAOA chondrocytes in 5 of 6 samples analyzed (figure 2D).



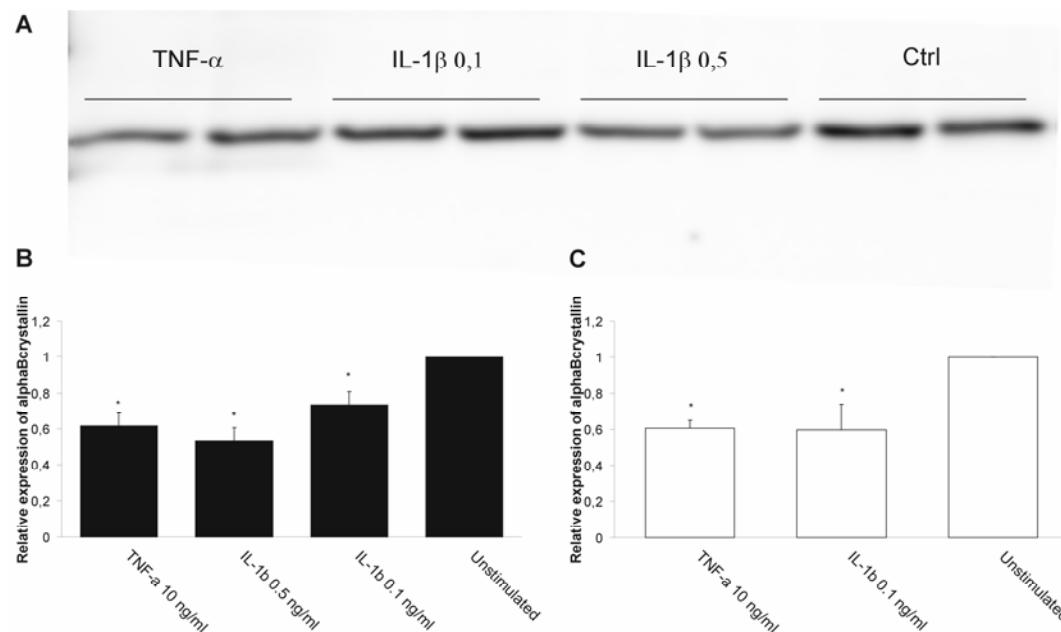


**Figure 2:** Expression of  $\alpha$ Bcrystallin in human articular chondrocytes isolated from healthy and OA-cartilage. **A:** The protein levels of  $\alpha$ Bcrystallin in extracts of chondrocytes isolated from healthy cartilage (left 5 lanes) and from visually intact (middle 4 lanes) and visually damaged (right 4 lanes) OA-cartilage were determined by Western blot analysis. Equal loading was verified using Ponceau S (data not shown). A gradual decrease in expression could be detected from NoNo to OAOA chondrocytes. **B:** Results of the densitometric analysis of the Western blots, showing the mean expression ( $\pm$  SEM) in each group (relative to NoNo expression levels). \*: Denotes statistical significance ( $p < 0,05$ ; Mann-Whitney U-test) compared to NoNo. **C:** Immunofluorescence microscopy images of OA chondrocytes. Upper image, green channel: monoclonal mouse IgG<sub>1</sub> anti- $\alpha$ Bcrystallin stained with AlexaFluor 488 labeled goat anti-mouse IgG<sub>1</sub>. Middle image, blue channel: DAPI nuclear stain. Lower image: Superimposition of green and blue channel. Scale bars indicate 10  $\mu$ m. **D:** Comparison of  $\alpha$ Bcrystallin expression at the mRNA level in chondrocytes isolated from 6 OA-patients. In 5 of 6 samples a marked reduction of  $\alpha$ Bcrystallin expression in OAOA samples was detected. mRNA levels were determined by quantitative real-time PCR. Expression levels have been calculated as the mean expression compared to 3 different household genes (GAPDH, HPRT and PPIA). Different signs represent the relative expression of each individual patient, normalized to OAOA samples.

### 3.4 The cytokines $IL-1\beta$ and $TNF-\alpha$ suppress the expression of $\alpha$ Bcrystallin

It is known that pro-inflammatory cytokines such as  $IL-1\beta$  and  $TNF-\alpha$  modulate the chondrocyte's metabolism and may play an important role in OA-pathogenesis. To determine if the expression of  $\alpha$ Bcrystallin is influenced by pro-inflammatory cytokines, chondrocytes isolated from NoOA and OAOA samples were treated with  $IL-1\beta$  and  $TNF-\alpha$ . In both cases, the pro-inflammatory cytokines suppressed the expression of  $\alpha$ Bcrystallin. Figure 3 shows the

dose-dependent suppression of  $\alpha$ Bcrystallin expression by IL-1 $\beta$ . No significant differences were found between NoOA and OAOA chondrocytes in response to 0,1 ng/ml IL-1 $\beta$  and TNF- $\alpha$ .



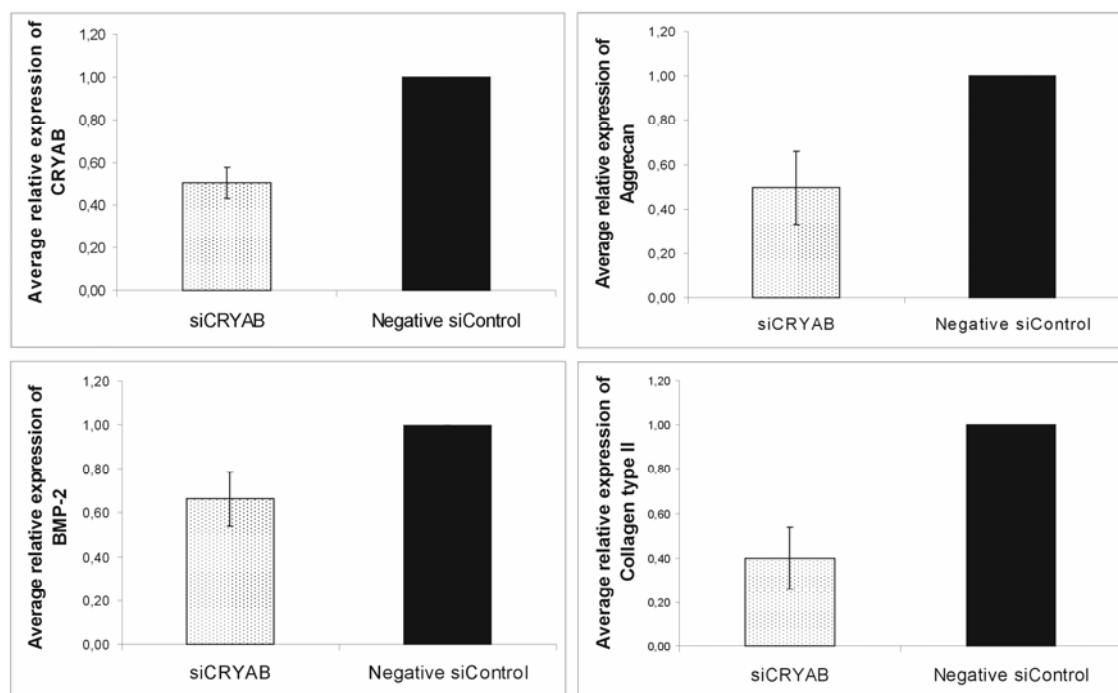
**Figure 3:** Relative expression of  $\alpha$ Bcrystallin as determined by Western blot. **A:** representative Western blot of chondrocytes isolated from NoOA-cartilage and stimulated with pro-inflammatory cytokines. From left to right: TNF- $\alpha$  (10 ng/ml); IL-1 $\beta$  (0.1 ng/ml); IL-1 $\beta$  (0,5 ng/ml); Unstimulated controls. Each stimulation was performed in duplicate. **B-C:** results of densitometric analysis. NoOA chondrocytes were isolated from 4 patients and stimulated with IL-1 $\beta$  (0,1 ng/ml – 0,5 ng/ml) or TNF- $\alpha$  (10ng/ml); OAOA chondrocytes were stimulated with IL-1 $\beta$  (0,1 ng/ml) or TNF- $\alpha$  (10ng/ml). Each stimulation was performed in duplicate per patient. Bars represent the average of the relative expression of 4 patients  $\pm$  SEM. Levels are normalized to unstimulated controls for NoOA and OAOA samples; \* denotes statistical significance ( $p < 0.05$ ; Mann-Whitney U-test) compared to unstimulated controls. Black bars represent NoOA samples, white bars represent OAOA samples.

### 3.5 RNAi induced knock-down of $\alpha$ Bcrystallin expression by siRNA results in decreased expression of chondrocyte-specific markers

A siRNA sequence against  $\alpha$ Bcrystallin (siCRYAB) was transfected in cultured chondrocytes. Non-silencing siRNA was used as a negative control. As shown in figure 4, siRNA transfection resulted in a marked reduction of  $\alpha$ Bcrystallin expression in CRYAB-siRNA samples compared to negative controls. In parallel, a second sequence was transfected, showing similar results (data not shown).

To explore the effects of a reduced expression of  $\alpha$ Bcrystallin on chondrocytes, the gene expression of 2 matrix genes (collagen type II and aggrecan) and the growth-factor, BMP-2,

was compared between silenced and non-silenced chondrocytes by real-time PCR. Figure 4 shows the average expression  $\pm$  SEM (4 experiments) of collagen type II, aggrecan and BMP-2 in silenced and non-silenced samples. For the siCRYAB sequence, there is a marked reduction in gene expression for these genes compared to non-silenced controls. Samples transfected with the second sequence showed similar trends (data not shown).

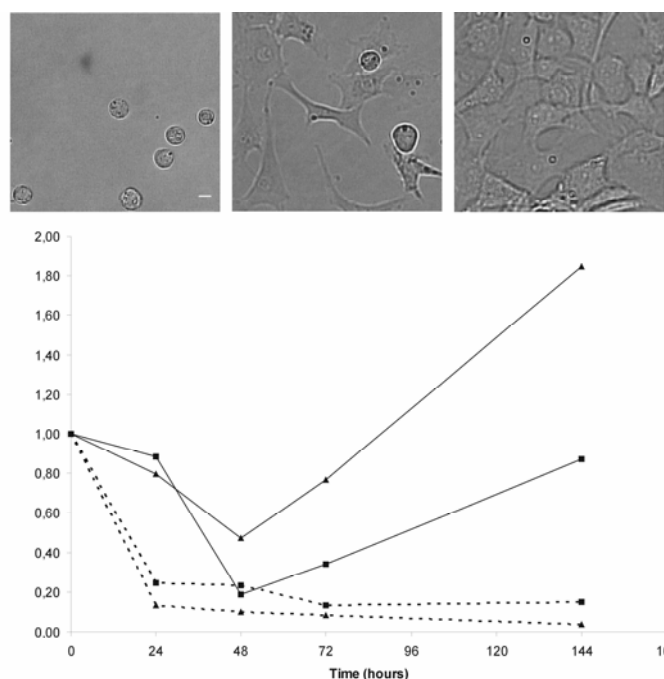


**Figure 4:** Expression levels of CRYAB, Aggrecan, BMP-2 and COL2A1 as determined by real-time PCR. Expression of CRYAB was suppressed by 21-mer siRNA sequences (siCRYAB). Non-silencing siRNA (Negative siControl) was used as a control. Expression levels have been calculated as the mean expression compared to 3 different household genes (GAPDH, HPRT and PPIA) in 4 different patients, 48 hours after transfection. Bars represent the average relative expression  $\pm$  SEM, normalized to Negative siControl.

### 3.6 *Morphologic dedifferentiation of chondrocytes is associated with a change in expression of $\alpha$ Bcrystallin*

Based on the observation that a decreased expression of  $\alpha$ Bcrystallin is associated with a reduced expression of chondrocyte differentiation markers, further experiments were conducted to evaluate the expression of  $\alpha$ Bcrystallin in dedifferentiating cells. Chondrocytes isolated from 2 OA-patients were seeded in monolayer cultures at low density (30.000 cells/cm<sup>2</sup>). As expected, this resulted in a quick morphologic change. Cell cultures were imaged at three different time points (Figure 5, upper panel) using a AxioVert 200 M (Carl Zeiss) microscope. At time of seeding, cells showed the typical rounded cell-shape associated with chondrocytes. After 144 hours almost all cells are attached to the surface and showed a

fibroblast-like morphology. Moreover, near confluent cell cultures were observed at this time point. The dedifferentiation was further confirmed by a strong decrease in the expression of BMP-2. This morphologic dedifferentiation is characterized by an initial decrease in expression of  $\alpha$ Bcrystallin. Between 72 and 144 hours, an increasing expression was observed.



**Figure 5:** Upper panel: Chondrocytes were seeded at low density. Cell cultures were imaged at different time points to allow visualization of morphologic dedifferentiation. From left to right: 0 h, 72 h and 144 h. Scale bars represent 10 $\mu$ m. Lower panel: Relative expression of  $\alpha$ Bcrystallin during dedifferentiation of chondrocytes seeded in monolayer at low density. Solid lines represent the expression of  $\alpha$ Bcrystallin at 0, 24, 48, 72 and 144 hours, indicating an initial decreased expression during dedifferentiation. Dashed lines represent the expression of the differentiation marker BMP-2 at 0, 24, 48, 72 and 144 hours, confirming that the cells are dedifferentiating during the experiment. Expression levels of  $\alpha$ Bcrystallin and BMP-2 are average relative expression levels normalized to three household genes (GAPDH, HPRT, PPIA). ■: patient 1; ▲patient 2.

#### 4. Discussion

This report describes the differential expression of  $\alpha$ Bcrystallin in OA-affected chondrocytes. RNAi gene knock down of  $\alpha$ Bcrystallin, indicates a role for this small heat shock protein as a novel mediator of the gene expression of matrix genes in chondrocytes.

The study was initiated by a gel-based proteome analysis, which revealed the differential abundance between healthy and OA chondrocytes of a protein, now identified as  $\alpha$ Bcrystallin. Subsequent Western blot analysis clearly showed the gradual decrease in abundance from normal to OA chondrocytes at the protein level. In addition to a differential abundance at the protein level, the mRNA levels of  $\alpha$ Bcrystallin are decreased in OAOA chondrocytes compared to NoOA chondrocytes, indicating that the differential expression is regulated at the

transcription level and is not a consequence of proteolytic cleavage or cellular translocation. It is known that  $\alpha$ Bcrystallin might translocate from the cytoplasm to the nucleus [23]. However, immunofluorescence microscopy data illustrate that  $\alpha$ Bcrystallin is mainly present in the cytoplasm of cultured chondrocytes.

IL-1 $\beta$  and TNF- $\alpha$  are the most prominent catabolic cytokines, which are synthesized by the chondrocyte itself and are generally thought to be involved in OA-pathogenesis. Interestingly, the protein levels of  $\alpha$ Bcrystallin in chondrocytes are cytokine-dependent (figure 3). These data suggest that the observed differential expression of  $\alpha$ Bcrystallin between healthy and OA chondrocytes appears to be regulated by a cytokine-driven mechanism,. In consequence,  $\alpha$ Bcrystallin might be presented as a novel potential down-stream mediator of the effects of IL-1 $\beta$  and TNF- $\alpha$  on human articular chondrocytes. Further research will be needed to explore the relation between these cytokines and  $\alpha$ Bcrystallin and to unravel the importance of this relationship in the pathogenesis of OA.

The differential expression of the CRYAB gene in OA chondrocytes and the sensitivity to inflammatory cytokines indicate a role for this gene as a potential mediator during the development of OA. This hypothesis is supported by a recent gene expression profiling study of articular cartilage in a rat model for early OA. The supplementary table of this report mentions a lower expression of CRYAB gene expression in rats with surgically induced OA compared to sham operated controls [24]. In addition, an  $\alpha$ Bcrystallin knockout model has been developed to study the role of this gene in lens structure and function [25]. Interestingly, the authors reported the display of a hunched posture in 12-month-old knockout mice which was associated with degenerative osteoarthritis of the intervertebral facet joints. Based on our own data, combined with the observations from the knockout model and the rat OA-model [24], a potential role in the pathophysiology of OA for this gene is very plausible.

However, no data were available pointing to a relationship between  $\alpha$ Bcrystallin and processes involved in cartilage metabolism. To explore the potential role of  $\alpha$ Bcrystallin in the chondrocyte's biology and to disclose the potential involvement in the pathogenesis of OA, we suppressed the expression of  $\alpha$ Bcrystallin by RNA interference (RNAi). RNAi is a naturally occurring phenomenon, which may be used to silence gene expression in mammalian cells by transfecting short double-stranded RNA sequences into the cells [26]. This results in a selective and efficient reduction in mRNA levels of the targeted gene (reviewed in [27]). In this report, we used low concentrations of different siRNA sequences

(5 nM, final concentration), thereby largely avoiding the possibility of off-target effects [28]. Even with this low concentration of siRNA we were able to suppress the expression of  $\alpha$ Bcrystallin in an efficient and reproducible way. Hereby demonstrating the ability to achieve a sufficient knock-down with low quantities of siRNA in cells which synthesize an ECM, such as chondrocytes. Under the chosen experimental conditions, the resulting average knock-down efficiency (50%) was comparable with the average reduction in mRNA expression between NoOA and OAOA samples (47%). This diminished expression was accompanied by a decreased expression in bone morphogenetic protein-2 (BMP-2) (figure 4), a member of the TGF- $\beta$  superfamily. It has been shown that the expression of collagen type II (COL2A1) as well as aggrecan is induced by rhBMP-2 in alginate embedded chondrocytes [29]. Not surprisingly, the expression of the matrix genes aggrecan and collagen type II showed a similar trend as BMP-2 expression, in samples with a reduced  $\alpha$ Bcrystallin gene-expression. These findings further add to the evidence of the involvement of  $\alpha$ Bcrystallin in the chondrocyte's biology.

COL2A1 and BMP-2 have previously been reported as markers of a stable chondrocyte phenotype [30]. BMP-2 is known as a promoter of chondrogenic differentiation in multipotent mesenchymal cells [31]. Moreover, it is generally known that dedifferentiation of chondrocytes is associated with phenotypic alterations and a reduced expression of cartilage-specific anabolic genes such as aggrecan and collagen type II [32]. Thus, the reduced expression of Aggrecan, COL2A1 and BMP-2 may reflect that a decreased expression of  $\alpha$ Bcrystallin drives the chondrocytes into a more dedifferentiated cell-type. This hypothesis is supported by the fact that  $\alpha$ Bcrystallin showed an initial decreased expression in dedifferentiating chondrocytes (figure 5). An inverse observation was made during the differentiation of the chondroprogenitor ATDC5 celline [10]: The differentiation of these cells into a chondrocyte-like phenotype, was associated with an initial increase in the expression of  $\alpha$ Bcrystallin. Furthermore, differential expression of  $\alpha$ Bcrystallin during differentiation processes has also been reported in C2C12 cells [9]. Our data and the data of Chen et al [10], implicate a potential role of  $\alpha$ Bcrystallin in the (de)differentiation processes of chondrocytes. Based hereon, it may be hypothesized that the increased expression of this gene in healthy chondrocytes may be associated with the differentiated and/or the rather metabolic inactive phenotype of healthy chondrocytes compared to the diseased counterparts.

In the RNAi-experiments, we used chondrocytes isolated from visually intact cartilage from OA-patients, which is more easily available than healthy articular cartilage. It is known that such chondrocytes show an altered phenotype compared to chondrocytes isolated from normal cartilage [33]. Whether the same relationship exists in healthy chondrocytes between  $\alpha$ Bcrystallin and chondrocyte-specific genes remains to be elucidated. Nevertheless, our experiments clearly indicate a functional link between  $\alpha$ Bcrystallin expression and chondrocyte-specific genes in OA-affected chondrocytes.

In conclusion, our study clearly demonstrates the differential expression of  $\alpha$ Bcrystallin at different levels between healthy and OA-affected articular chondrocytes. This differential expression seems to be a consequence of a cytokine driven mechanism. Additionally, our data disclose  $\alpha$ Bcrystallin as a novel identified potential regulator of matrix gene expression. The association between reduced  $\alpha$ Bcrystallin levels and reduced levels of aggrecan, collagen type II and BMP-2, further adds to the evidence that this protein might be involved in phenotypic changes of chondrocytes during the development of OA.

## Acknowledgements

We wish to thank Dr. P. Verdonk and Dr. K.F. Almqvist for providing cartilage samples.

## 5. REFERENCES

- [1] van den, I. P., Norman, D. G., Quinlan, R. A., Molecular chaperones: small heat shock proteins in the limelight. *Curr Biol* 1999, 9, R103-105.
- [2] Haslbeck, M., Franzmann, T., Weinfurter, D., Buchner, J., Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol* 2005, 12, 842-846.
- [3] Djabali, K., Piron, G., de Nechaud, B., Portier, M. M., alphaB-crystallin interacts with cytoplasmic intermediate filament bundles during mitosis. *Exp Cell Res* 1999, 253, 649-662.
- [4] Perng, M. D., Cairns, L., van den, I. P., Prescott, A., *et al.*, Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. *J Cell Sci* 1999, 112 (Pt 13), 2099-2112.
- [5] Singh, B. N., Rao, K. S., Ramakrishna, T., Rangaraj, N., Rao Ch, M., Association of alphaB-Crystallin, a Small Heat Shock Protein, with Actin: Role in Modulating Actin Filament Dynamics in Vivo. *J Mol Biol* 2007, 366, 756-767.
- [6] Kamradt, M. C., Lu, M., Werner, M. E., Kwan, T., *et al.*, The Small Heat Shock Protein {alpha}B-crystallin Is a Novel Inhibitor of TRAIL-induced Apoptosis That Suppresses the Activation of Caspase-3. *J Biol Chem* 2005, 280, 11059-11066.
- [7] Kamradt, M. C., Chen, F., Cryns, V. L., The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem* 2001, 276, 16059-16063.
- [8] Mehlen, P., Kretz-Remy, C., Preville, X., Arrigo, A. P., Human hsp27, Drosophila hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *Embo J* 1996, 15, 2695-2706.
- [9] Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Kato, K., Regulation of the levels of small heat-shock proteins during differentiation of C2C12 cells. *Exp Cell Res* 2001, 266, 213-221.
- [10] Chen, L., Fink, T., Zhang, X. Y., Ebbesen, P., Zachar, V., Quantitative transcriptional profiling of ATDC5 mouse progenitor cells during chondrogenesis. *Differentiation* 2005, 73, 350-363.
- [11] Favet, N., Duverger, O., Loones, M. T., Poliard, A., *et al.*, Overexpression of murine small heat shock protein HSP25 interferes with chondrocyte differentiation and decreases cell adhesion. *Cell Death Differ* 2001, 8, 603-613.
- [12] Vicart, P., Caron, A., Guicheney, P., Li, Z., *et al.*, A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat Genet* 1998, 20, 92-95.
- [13] Berry, V., Francis, P., Reddy, M. A., Collyer, D., *et al.*, Alpha-B crystallin gene (CRYAB) mutation causes dominant congenital posterior polar cataract in humans. *Am J Hum Genet* 2001, 69, 1141-1145.
- [14] Sun, Y., MacRae, T. H., The small heat shock proteins and their role in human disease. *Febs J* 2005, 272, 2613-2627.



- [15] Goldring, M. B., The role of the chondrocyte in osteoarthritis. *Arthritis Rheum* 2000, *43*, 1916-1926.
- [16] Aigner, T., Kurz, B., Fukui, N., Sandell, L., Roles of chondrocytes in the pathogenesis of osteoarthritis. *Curr Opin Rheumatol* 2002, *14*, 578-584.
- [17] Lambrecht, S., Verbruggen, G., Verdonk, P., Elewaut, D., Deforce, D., Differential proteome analysis of normal and osteoarthritic chondrocytes reveals distortion of vimentin network in osteoarthritis. *Osteoarthritis Cartilage*, doi: 10.1016/j.joca.2007.06.005.
- [18] Verbruggen, G., Wang, J., Wang, L., Elewaut, D., Veys, E. M., *Analysis of chondrocyte functional markers and pericellular matrix components by flow cytometry*, Humana Press, Totowa, New Jersey 2004.
- [19] Guo, J. F., Jourdian, G. W., MacCallum, D. K., Culture and growth characteristics of chondrocytes encapsulated in alginate beads. *Connect Tissue Res* 1989, *19*, 277-297.
- [20] Verbruggen, G., Veys, E. M., Wieme, N., Malfait, A. M., *et al.*, The synthesis and immobilisation of cartilage-specific proteoglycan by human chondrocytes in different concentrations of agarose. *Clin Exp Rheumatol* 1990, *8*, 371-378.
- [21] Livak, K. J., Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-</sup>(Delta Delta C(T)) Method. *Methods* 2001, *25*, 402-408.
- [22] Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., *et al.*, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002, *3*, 34.
- [23] van den, I. P., Wheelock, R., Prescott, A., Russell, P., Quinlan, R. A., Nuclear speckle localisation of the small heat shock protein alpha B-crystallin and its inhibition by the R120G cardiomyopathy-linked mutation. *Exp Cell Res* 2003, *287*, 249-261.
- [24] Appleton, C. T., Pitelka, V., Henry, J., Beier, F., Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 2007, *56*, 1854-1868.
- [25] Brady, J. P., Garland, D. L., Green, D. E., Tamm, E. R., *et al.*, AlphaB-crystallin in lens development and muscle integrity: a gene knockout approach. *Invest Ophthalmol Vis Sci* 2001, *42*, 2924-2934.
- [26] Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., *et al.*, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, *411*, 494-498.
- [27] Novina, C. D., Sharp, P. A., The RNAi revolution. *Nature* 2004, *430*, 161-164.
- [28] Persengiev, S. P., Zhu, X., Green, M. R., Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *Rna* 2004, *10*, 12-18.
- [29] Grunder, T., Gaissmaier, C., Fritz, J., Stoop, R., *et al.*, Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. *Osteoarthritis Cartilage* 2004, *12*, 559-567.

- [30] Dell'Accio, F., De Bari, C., Luyten, F. P., Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. *Arthritis Rheum* 2001, *44*, 1608-1619.
- [31] Majumdar, M. K., Wang, E., Morris, E. A., BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. *J Cell Physiol* 2001, *189*, 275-284.
- [32] Sandell, L. J., Aigner, T., Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 2001, *3*, 107-113.
- [33] Wang, J., Verdonk, P., Elewaut, D., Veys, E. M., Verbruggen, G., Homeostasis of the extracellular matrix of normal and osteoarthritic human articular cartilage chondrocytes in vitro. *Osteoarthritis Cartilage* 2003, *11*, 801-809.

## **Chapter III: Results**

*Part 3: The proteome of human articular chondrocytes explored by a shotgun proteomics approach*



# The proteome of human articular chondrocytes explored by a shotgun proteomics approach

Stijn Lambrecht<sup>1</sup>, Maarten Dhaenens<sup>1</sup>, Gust Verbruggen<sup>2</sup>, Dirk Elewaut<sup>2</sup>, Dieter Deforce<sup>1</sup>

<sup>1</sup>Laboratory of Pharmaceutical Biotechnology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium; <sup>2</sup>Department of Rheumatology, University Hospital Ghent, B-9000 Ghent, Belgium;

**Corresponding author:** Prof. Dieter Deforce; Laboratory of Pharmaceutical Biotechnology, Harelbekestraat 72, B-9000 Ghent, Belgium; e-mail: [dieter.deforce@ugent.be](mailto:dieter.deforce@ugent.be); Fax: +32-9-220-6688

**To be submitted for publication**

## Abstract

*Objective:* In recent years, some studies have been initiated to disclose the proteome of human chondrocytes and cartilage. Despite these studies, comprehensive information of the chondrocyte proteome remains limited. Especially the membrane and low-abundance proteome remain largely unknown. This study aims to dig further in the proteome expressed by human knee chondrocytes, thereby providing a database for future biomolecular research.

*Methods:* Chondrocytes isolated from human knee cartilage samples were cultured in a three-dimensional alginate culture system. To simplify the protein mixtures, proteins extracted from chondrocyte cell lysates were fractionated based on hydrophobicity and molecular weight. Proteins were digested and the resulting peptides were separated and identified by an on-line 2-D nanoLC system coupled to a Qq-TOF mass spectrometer.

*Results:* Using extensive prefractionation, several membrane proteins, transcription factors and other low-abundance proteins could be identified. Moreover, low molecular weight proteins, generally lost in a gel-based approach, could be enriched and identified. Several proteins, known to play an important role in chondrocyte homeostasis and OA-pathogenesis, such as tissue inhibitors of matrix metalloproteinases and IGF-binding proteins could be reliably identified. On the other hand, proteins which are related to cytokine/growth factor pathways and which have never been described in chondrocytes could be detected. In addition, novel proteins related to known disease pathways or disease processes were disclosed. The aim of the chondrocyte proteome database was illustrated by the rational selection of HSP27, a protein related to the previously described  $\alpha$ Bcrystallin, for further differential expression analysis.

*Conclusion:* Extensive prefractionation of proteins followed by 2-D nanoLC coupled to mass spectrometry allows to dig deeper in the chondrocyte proteome. This study is a first exploration of the 'hidden' proteome of articular chondrocytes and may serve as a reference tool for future biomolecular research.

## 1. Introduction

Osteoarthritis (OA) has become one of the major health problems in the western world [1]. The key hallmark of this disease is a slow progressive degeneration of the articular cartilage [2]. Articular cartilage forms a specialized, smooth connective tissue that is weight bearing and that serves as a gliding surface allowing a lithe movement of the joints. Cartilage is a very attractive tissue as it contains only one single cell type. However, for proteomics studies this forms a major challenge, as this cell type comprises only 1% of the cartilage volume and the majority of the tissue is formed by highly abundant extracellular-matrix (ECM) components [3]. As it is generally accepted that disturbances in chondrocyte metabolism are involved in the pathogenesis of OA [4], the knowledge of the chondrocyte- as well as the cartilage-proteome may provide a new tool, which may assist in the elucidation of pathways involved in cartilage destruction associated with OA.

In recent years, several attempts have been performed to tackle the chondrocyte and/or cartilage proteomes. An extensive prefractionation method followed by 2-DE gel separation was proposed by Vincourt et al [5]. A limited number of unique proteins were identified by MALDI-TOF mass spectrometry of gel-separated spots. A 1-D SDS-PAGE approach combined with tandem mass spectrometry, resulted in the identification of 100 different proteins from knee cartilage specimens [6]. A 2-DE based approach was used to identify proteins of monolayer cultured human articular chondrocytes by Ruiz-Romero et al. [7], yielding a set of 93 unique proteins. In a subsequent study, the same group initiated the proteome characterization of mitochondria of chondrocytes, resulting in the identification of 49 unique proteins [8]. Despite these studies, there is some lack of comprehensive information from cartilage tissue as well as isolated chondrocytes. More specific, the ‘hidden’ proteome which is not accessible by standard 2-DE methods, is poorly characterized.

Prefractionation based on hydrophobicity is a well known sample preparation method in 2-D gel applications to achieve more simplified spot patterns [9]. Whereas 2-DE remains very attractive for differential expression analysis [10], 2-D LC methods have become the method of choice for the large-scale characterization of a given proteome. In this study, we combine such 2-D LC MS-approach with prefractionation methods to dig deeper in the proteome of human chondrocytes.

## 2. Materials and Methods

### 2.1 *Isolation and culture of chondrocytes*

Human articular knee cartilage was obtained from 4 donors (one male, three female, median age  $\pm$  SD:  $70 \pm 10,9$  years) after total knee arthroplasty. The study protocol was approved by the local Ethics Committee. The cartilage was diced into small fragments and chondrocytes were isolated by sequential enzymatic digestion (hyaluronidase, pronase, collagenase (Sigma-Aldrich, Steinheim, Germany)) as described in detail elsewhere [11]. Trypan blue exclusion revealed that >95% of the cells were viable after isolation.

Chondrocyte cultures in alginate beads were prepared and maintained as described previously [12]. For each sample,  $15\text{--}17,5 \times 10^6$  chondrocytes were lysed and treated as described below.

### 2.2 *Sample preparation and protein digestion*

*Total cell lysate*- Isolated chondrocytes were resuspended and lysed in a denaturing buffer containing 8 M urea. After reduction and alkylation, proteins were digested with sequencing grade trypsin at 37°C for 12 to 16 h. Peptide solutions were desalted using solid-phase extraction cartridges (Oasis®, Waters, Milford, USA), dried in a vacuum-centrifuge and frozen at -80°C until use.

*Fractionation based on hydrophobicity and Mw*- Isolated chondrocytes were resuspended in Reagent 1 (40mM Tris) from the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA), containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and a phosphatase inhibitor-cocktail (Sigma-Aldrich, Steinheim, Germany). Cells were lysed by sonication and the proteins were extracted using the ReadyPrep Sequential Extraction Kit (Bio-Rad) according to the manufacturer's protocol. This resulted in a soluble, an intermediate and a hydrophobic protein fraction [9]. All fractions were dialysed using 2 kDa Mw cut-off centrifugal devices (Vivaspin, Vivascience). The resulting concentrates were diluted in dissolution buffer (iTRAQ reagents kit, Applied Biosystems) supplemented with a SDS-containing denaturant solution. After reduction and alkylation, the intermediate and hydrophobic fractions were digested using sequencing grade trypsin. The soluble fraction was further fractionated, based on molecular weight using 300 kDa, 100 kDa, 10 kDa and 2 kDa Mw cut-off centrifugal devices (Vivaspin, Vivascience). Each resulting concentrate was digested as described above. For a second chondrocyte sample, the resulting pellet after the second extraction, was resuspended in a 2% SDS-solution containing 5%  $\beta$ -mercaptoethanol. The suspension was heated at 95°C for 5 minutes. The sample was cooled to room



temperature and centrifuged at 15000 rpm. The resulting supernatans was subjected to 1-D gelelectrophoresis. Precision Plus All Blue standards (Bio-Rad) were run in parallel as molecular weight markers. The gel was stained with Colloidal Coomassie blue (Pierce). The lane was divided in eight zones and each zone was isolated and cut in smaller fragments followed by in-gel digestion as described previously [12], except that larger volumes were used.

### **2.3    *On-line two-dimensional nanoLC-MS***

Peptides were resuspended in buffer A (Buffer A: 2,5 mM NaH<sub>2</sub>PO<sub>4</sub>, 3% ACN, pH=2,7). For ESI Qq-ToF analysis the resuspended peptides were injected on an Ultimate 3000 autosampler (LC Packings, Sunnyvale, CA, USA) and trapped on a Poros 10S strong-cation exchange (SCX) column (300 µm i.d. x 15 cm). Peptides were eluted from the SCX column by a 840 min discontinuous gradient from 0 to 100 % buffer B (Buffer B: 2,5 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 3% ACN, pH=2,7, flow rate= 6 µl/min). Two reversed-phase trap-columns (PepMap, LC Packings) were used for parallel trapping of the peptides, eluting from the SCX column, prior to the separation on a C18 PepMap column (LC Packings) by a 70 min linear gradient from 6 to 100 % buffer C (80% acetonitrile and 0,1% formic acid in water). A Switchos pump (LC Packings) [pumping 12 µl/min buffer D (0,1% formic acid in water)] was coupled to the LC-system to wash the trap columns prior to peptide elution. ESI Qq-ToF analysis was performed on a Q-ToF Ultima mass spectrometer (Waters, Milford USA), which was coupled to the LC-system via a nano-LC inlet. The instrument was calibrated using fragment ions generated from MS/MS of Glu-fibrinopeptide B (Sigma-Aldrich).

### **2.4    *Database searching***

The Q-TOF was operated in a data-dependent mode by performing MS/MS scans for the 7 the most intense peaks from each MS scan. The MS scan range was 450-1100 m/z. Peak list files were generated from the raw data by Mascot Distiller version 2.1.0.0 (Matrix Science). The generated files were merged into a single .mgf file. Database searches against the Swiss-Prot database (version 51.6) were performed using an in-house licensed Mascot search engine (version 2.2.0.0). The search parameters were: a maximum of 1 missed cleavages using trypsin; fixed modifications were carbamidomethylation and variable modifications were oxidation of methionine and N-terminal acetylation; the mass tolerances were set to 0.20 and 0.15 Da for precursor ions and fragment ions respectively; one C13 was allowed. Only protein identifications with a p-value less than 0,01 were accepted. Protein identifications based on a

single peptide hit were manually validated and only accepted if three consecutive y or b ions matched intense peaks in the MS/MS-spectrum.

## **2.5 Western Blot analysis**

Chondrocyte samples of 6 healthy donors (4 male, 2 female, median age  $\pm$  SD: 46,5  $\pm$  23,6 years) and 6 OA patients (3 male, 3 female, median age  $\pm$  SD: 66,5  $\pm$  11,1 years) were collected, isolated and cultured as described previously [13]. Cell lysates of chondrocytes were prepared by resuspending cell pellets in 40mM Tris from the ReadyPrep Sequential Extraction Kit (Bio-Rad, Hercules, CA, USA), containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and a phosphatase inhibitor-cocktail (Sigma-Aldrich, Steinheim, Germany). Cells were lysed by sonication and the soluble proteins were isolated by centrifugation. Equal amounts (30  $\mu$ g as determined by 2-D Quant kit, GE Healthcare, Fairfield, USA) of soluble fractions of 18 samples [6 NoNo (chondrocytes isolated from healthy cartilage), 6 NoOA (chondrocytes isolated from visually intact cartilage of OA-patients) and 6 OAOA (chondrocytes isolated from visually damaged zones of OA-cartilage)] were loaded on 10% SDS-PAGE gels. Equal loading was verified by Ponceau S staining (data not shown). MagicMark (Invitrogen, Paisley, UK) protein standards were run as molecular weight markers. Following 1-D gel electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad). The resulting membranes were immunostained with a mouse monoclonal anti-HSP27 (G3.1) (Abcam, Cambridge, UK) followed by an anti-mouse HRP-conjugated secondary antibody (Pierce, Rockford, IL, USA) and enhanced chemiluminescence detection (Supersignal West Dura Extended Duration Substrate, Pierce). Chemiluminescence images were recorded using the VersaDoc-imaging system (Bio-Rad). Image analysis was performed by Quantity One v 4.4.0 (Bio-Rad).

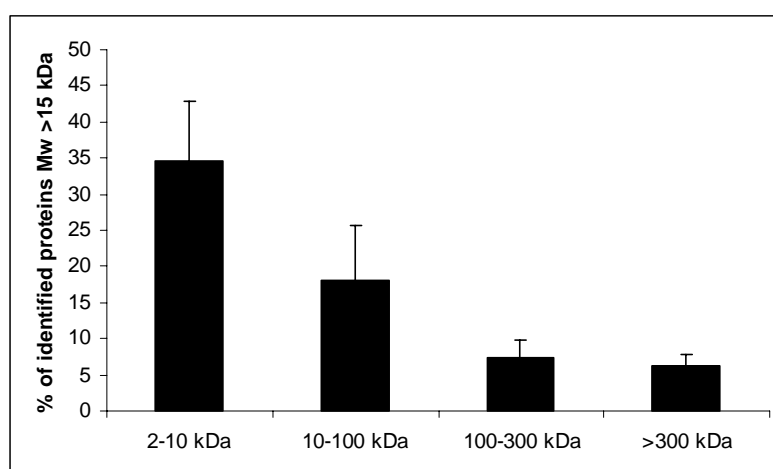
## **3. Results and Discussion**

### ***Prefractionation based on hydrophobicity and molecular weight yields a higher number of identifications***

Fractionation based on hydrophobicity is a well-known method to simplify spot patterns in a 2-DE based proteomics approach [9]. In this study, such approach was used as prefractionation tool to explore the chondrocyte proteome. In the unfractionated samples, 379 unique proteins could be identified. Using prefractionation based on the hydrophobicity and the molecular weight of the proteins this number was extended to 717. As could be expected,

prefractionation based on hydrophobicity results in the enrichment of membrane proteins in the hydrophobic fractions. Whereas 40,6 % of the identified proteins were annotated to the membrane (as assigned by the GeneOntology database, [www.geneontology.org](http://www.geneontology.org)) in the hydrophobic fractions, this was only 22,6 % in the soluble fractions.

Next to hydrophobicity, the molecular weight of the proteins was used as a second prefractionation tool. Low molecular weight proteins might be expected to be underrepresented in a shotgun proteomics approach, given the fact that a lower number of identifiable peptides per protein are generated. To increase the number of protein identifications with a low molecular weight, we combined the sequential extraction with prefractionation of the soluble proteome by ultrafiltration. The four resulting fractions (theoretical Mw-ranges: 2-10, 10-100, 100-300, >300 kDa) were analysed separately by LC-MS. Figure 1 indicates an enrichment of low molecular weight proteins (<15 kDa) in the 2-10 kDa fraction. However, in this fraction a considerable number of identified proteins had a theoretical molecular weight which is higher than the predicted cut-off. It may be assumed that, next to the nature of filtration membranes, phenomena such as protein post-translational modifications will also contribute to this observation.

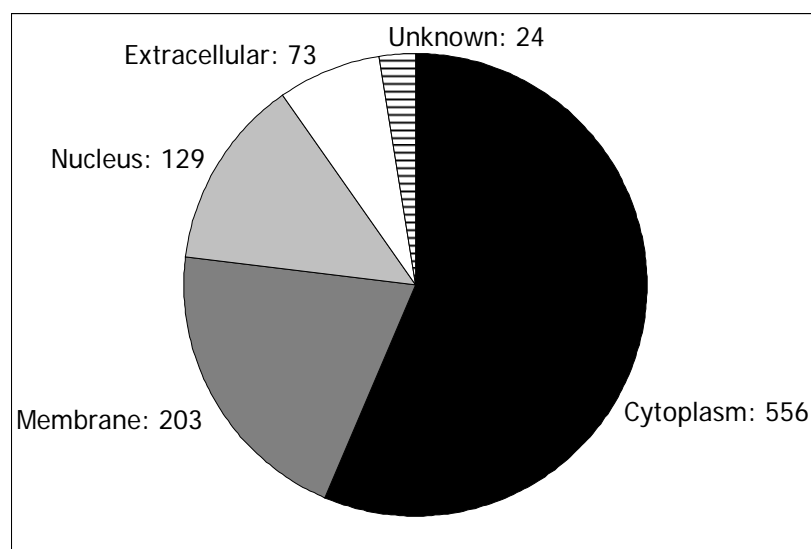


**Figure 1:** % of protein identifications which show a theoretical molecular weight (molecular weight of the unprocessed precursor) smaller than 15 kDa. The soluble proteome was fractionated based on ultrafiltration in four different fractions: 2-10 kDa, 10-100 kDa, 100-300 kDa, >300 kDa, resulting in an enrichment of low molecular weight proteins. Bars indicate mean %  $\pm$  SD.

### ***2-D nanoLC combined with tandem MS yields a “chondrocyte proteome database”***

The aim of this study was to provide a database which may assist in future molecular biology research in chondrocytes. Using extensive prefractionation followed by ESI-MS/MS, 779 unique proteins expressed by cultured chondrocytes could be reliably ( $p < 0,01$ ) identified. The

false discovery rate was estimated at 0,3 % by searching a reversed database. The complete list of identifications and the corresponding experimental data can be found in the supplementary table.



**Figure 1:** Pie chart showing the cellular localization of the identified proteins, as assigned by the GeneOntology database.

The cellular localization of the identified proteins was assigned by searching the GeneOntology database, indicating a diverse distribution of the identified proteins (see figure 2). 556 of the identified proteins are localized in the cytoplasm, 203 proteins were annotated to the membrane, 129 proteins to the nucleus and 73 are located in the extracellular environment. A gene ontology search on the molecular function indicated that 22 identified proteins are involved in signal transduction, whereas 7 proteins were identified with transcription factor activity. 14 proteins were identified with receptor activity and 32 transmembrane transporters were reported (see Table 1).

Several proteins have been identified, which are known to play an important role in chondrocyte homeostasis and OA-pathogenesis. For example, insulin-like growth factor binding proteins (IGFBP-7), tissue inhibitors of matrix metalloproteinases (TIMP-1) and matrix-metalloproteinases (MMP-3 and MMP-1).

Alternatively, several extracellular matrix proteins and proteins involved in ECM organization (such as the CD-44 receptor and  $\beta$ 1- and  $\beta$ 5-integrin) could be identified. As could be expected from chondrocytes cultured in alginate beads, collagens type I, II and VI were identified next to other well known matrix proteins among these the aggrecan core protein, biglycan, perlecan, the hyaluronan and proteoglycan link protein and chondroitin sulphate proteoglycan 4 precursor. The three different  $\alpha$  chains which form the heterotrimer type VI

collagen could all be identified with high sequence coverage ( $\alpha 1$ : 35%,  $\alpha 2$ : 26%,  $\alpha 3$ : 25%) indicating a relatively high abundance especially if compared to collagen type II (1% sequence coverage). However, it is known that type VI collagen forms only a small portion of the cartilage collagens [14]. This clearly indicates that during cell isolation after alginate culture the chondrocytes are selectively isolated with their cell-associated matrix from the interterritorial matrix, as the pericellular matrix is rich in type VI collagen and excludes type II collagen [15].

**Table 1:** Protein identifications which were assigned to the following GeneOntology-terms: transmembrane transporter activity, kinase activity, signal transducer activity, structural constituent of cytoskeleton, extracellular matrix structural constituent, receptor activity, antioxidant activity, transcription factor activity, transmembrane receptor activity.

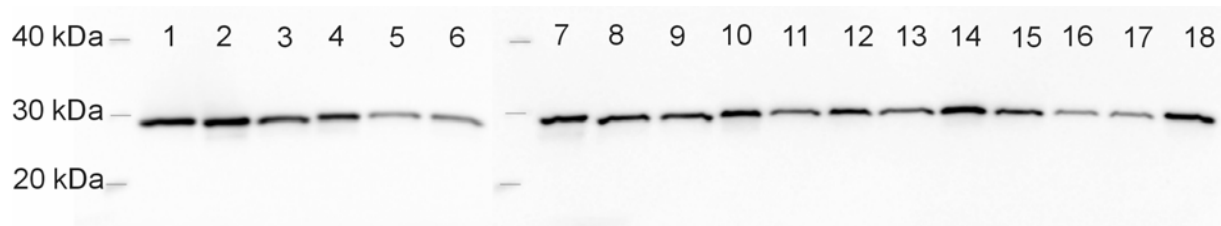
<b>Transmembrane transporter activity</b>	ADT1_HUMAN, ADT3_HUMAN, ANKH_HUMAN, ANXA7_HUMAN, AT1A1_HUMAN, ATOX1_HUMAN, ATPA_HUMAN, ATPB_HUMAN, ATPD_HUMAN, ATPK_HUMAN, ATPO_HUMAN, CERU_HUMAN, CLIC1_HUMAN, CLIC4_HUMAN, COX2_HUMAN, CX6B1_HUMAN, FRDA_HUMAN, KE4_HUMAN, MPCP_HUMAN, PEA15_HUMAN, S29A1_HUMAN, SC61G_HUMAN, TOM40_HUMAN, TTHY_HUMAN, TXTP_HUMAN, UCRH_HUMAN, UQCR1_HUMAN, VATB1_HUMAN, VATG1_HUMAN, VDAC1_HUMAN, VDAC2_HUMAN, VDAC3_HUMAN
<b>Kinase activity</b>	DAK_HUMAN, FRDA_HUMAN, HXK1_HUMAN, ILK_HUMAN, K6PL_HUMAN, K6PP_HUMAN, KAD1_HUMAN, KAD2_HUMAN, KAD3_HUMAN, KAPCA_HUMAN, KCRB_HUMAN, KCY_HUMAN, KPYM_HUMAN, NDKA_HUMAN, NDKB_HUMAN, PAPS1_HUMAN, PAPS2_HUMAN, PASK_HUMAN, PDXK_HUMAN, PGK1_HUMAN, PGK2_HUMAN, RBSK_HUMAN
<b>Signal transducer activity</b>	ARF1_HUMAN, CSPG4_HUMAN, EF1D_HUMAN, FLNA_HUMAN, GBB1_HUMAN, GNA12_HUMAN, GNAL_HUMAN, GRB2_HUMAN, HMOX1_HUMAN, IFM1_HUMAN, LEG1_HUMAN, PASK_HUMAN, PLCD1_HUMAN, RHG01_HUMAN, RHOA_HUMAN, RHOC_HUMAN, SAM68_HUMAN, SH3L1_HUMAN, STAT1_HUMAN, TSP1_HUMAN, VAPA_HUMAN

<b>Structural constituent of cytoskeleton</b>	ACTB_HUMAN, ACTG_HUMAN, ARC1B_HUMAN, E41L2_HUMAN, K1C16_HUMAN, K1C9_HUMAN, K22E_HUMAN, K2C1_HUMAN, MOES_HUMAN, PLEC1_HUMAN, SPTA2_HUMAN, SPTB2_HUMAN, TBB2A_HUMAN, TBB3_HUMAN, TLN1_HUMAN, VIME_HUMAN
<b>Extracellular matrix structural constituent</b>	CH3L1_HUMAN, CHAD_HUMAN, CO1A1_HUMAN, CO1A2_HUMAN, CO2A1_HUMAN, CO6A1_HUMAN, CO6A2_HUMAN, COMP_HUMAN, FINC_HUMAN, MGP_HUMAN, PGCA_HUMAN, PGS1_HUMAN, PRELP_HUMAN, RRB1_HUMAN
<b>Receptor activity</b>	AMRP_HUMAN, CD44_HUMAN, FKB1A_HUMAN, ICAM1_HUMAN, ITB1_HUMAN, ITB5_HUMAN, K2C1_HUMAN, LRP1_HUMAN, MEP50_HUMAN, MRC2_HUMAN, NPC1_HUMAN, RRB1_HUMAN, RSSA_HUMAN, SCRB2_HUMAN, TBB3_HUMAN, TMED7_HUMAN
<b>Antioxidant activity</b>	APOE_HUMAN, GPX1_HUMAN, PRDX1_HUMAN, PRDX2_HUMAN, PRDX3_HUMAN, PRDX4_HUMAN, PRDX5_HUMAN, PRDX6_HUMAN, SODC_HUMAN
<b>Transcription factor activity</b>	ENOA_HUMAN, HMGA1_HUMAN, HMGB2_HUMAN, NDKB_HUMAN, PA2G4_HUMAN, STAT1_HUMAN, YBOX1_HUMAN
<b>Transmembrane receptor activity</b>	AMRP_HUMAN, ICAM1_HUMAN, NPC1_HUMAN, RSSA_HUMAN

Oxygen and reactive oxygen species (ROS) play a crucial role in the control of cartilage homeostasis and oxidative stress has previously been related to the pathogenesis of OA (reviewed in [16]). Gene ontology searches showed the identification of 10 proteins with anti-oxidant activity, among these the peroxiredoxins. Peroxiredoxins are a family of proteins involved in oxidative stress defence in human cells. In previous studies peroxiredoxin-5 [17] and -6 [13] have been reported as differentially expressed proteins in OA-affected chondrocytes. Our data show the reliable identification of the other members of the peroxiredoxin-family (PRDX1, 2, 3 and 4). Given the reported differential expression of peroxiredoxin-5 and -6, this protein family absolutely merits further investigation.

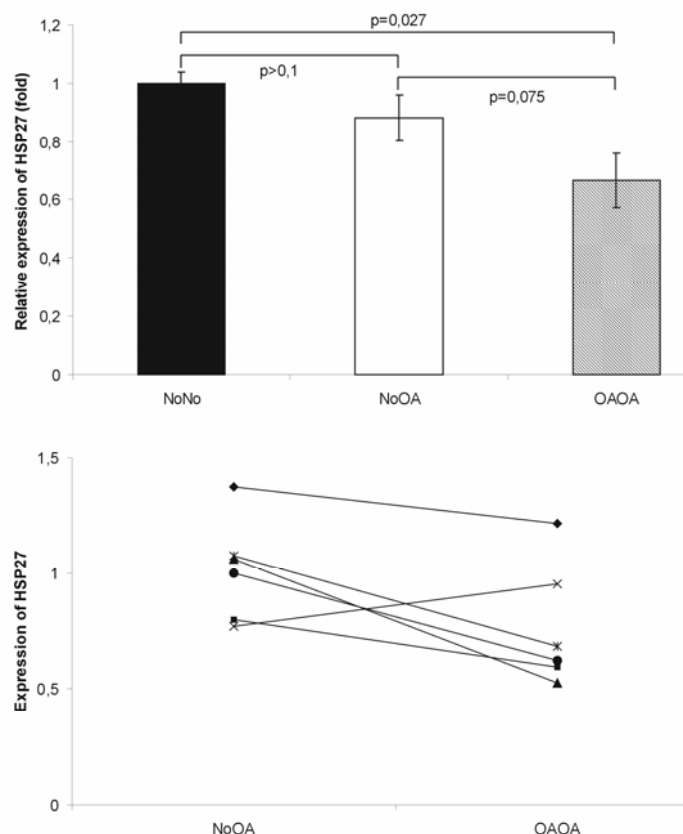
### ***Rational target selection from the database may provide novel potential players in OA-pathogenesis***

In a previous report we identified  $\alpha$ Bcrystallin as a potential interesting protein in OA pathogenesis (Chapter III, Part 2).  $\alpha$ Bcrystallin belongs to the family of small heat-shock proteins (sHSPs), a family of molecular chaperones with similar structures and functionalities [18]. Until now, 10 different sHSPs have been identified in human [19]. In our study, we identified three members of this family: the previously described  $\alpha$ Bcrystallin (HSPb5), HSP20 (HSPb6) and HSP27 (HSPb1). HSP27 and  $\alpha$ Bcrystallin are the most investigated sHSPs. Both are very closely related, in function as well as in structure (reviewed in [20]). Based hereon, we further examined the expression of HSP27 in OA-affected chondrocytes, revealing a reduced expression of this protein in cultured OA-chondrocytes. Our western blot data show a significant reduced expression of HSP27 in OA-chondrocytes compared to chondrocytes isolated from healthy cartilage (33 % reduction,  $p < 0,05$ -Mann Whitney U-test) (Figure 3 and 4). In 5 of the 6 patients analysed, HSP27 shows a higher expression in chondrocytes isolated from visually intact compared to visually damaged zones from the same knee joint.



**Figure 3:** Expression of HSP27 in human articular chondrocytes isolated from healthy and OA cartilage. The protein levels of HSP27 in extracts of chondrocytes isolated from healthy cartilage (lanes 1-2 and 7-10) and from visually intact (lanes 3-4 and 11-14) and visually damaged zones (lanes 5-6 and 15-18) of OA cartilage were determined by Western blot analysis. Equal loading was verified using Ponceau S (data not shown).

Further research will be needed to elucidate the true biological role of this differential expression in the pathogenesis of OA. However, based on current literature, a significant role may be suspected. In cell lines, HSP27 has been reported as a downstream effector of TGF- $\beta$  [21], a key growth factor in chondrocyte metabolism [22]. Moreover, it has been shown that overexpression of HSP25 (the murine analogue of HSP27) in C1 cell lines, interferes with chondrogenic differentiation of this cell line [23].



**Figure 4:** Densitometric analysis of Western blots reveals a differential expression between the sample groups. Upper panel: Results of the densitometric analysis, showing the mean expression ( $\pm$  SEM) in each group (normalized to NoNo expression levels). P-values were calculated using Mann-Whitney *U*-test for the comparison between NoNo vs NoOA and NoNo vs OAOA. NoOA and OAOA samples were compared by Wilcoxon's paired sample test. Black bars: NoNo; white: NoOA; shaded: OAOA. Lower panel: Results of the densitometric analysis, showing the expression levels of HSP27 in chondrocytes isolated from visually intact zones (NoOA) and visually damaged zones (OAOA) of the same knee joint from 6 different patients.

This study serves as a first exploration of the 'hidden' proteome of articular chondrocytes, resulting in the identification of several low-abundance and membrane proteins expressed by the chondrocyte. Therefore, a variety of sample preparation methods based on different protein and peptide properties were combined and applied to different samples. Based on current and future knowledge of important pathways and differentially abundant protein species, the provided database could assist in the rational selection of candidate experimental targets. This was demonstrated by the selection of HSP27, a protein closely related to the previously described  $\alpha$ Bcrystallin. Moreover, a more extended view on the chondrocyte's proteome may assist in the development of protein- as well as cDNA-arrays, which cover novel identified genes expressed by chondrocytes.

Furthermore, this report opens up perspectives to introduce a 'gel-free' comparative analysis between healthy and OA-chondrocytes. Further experiments are essential to select the most optimal workflow in terms of reproducibility and proteome coverage, thereby enabling differential proteome analyses based on labelling strategies.



## Acknowledgements

The authors greatly appreciate the excellent technical assistance of S. Vandecasteele with the MS-experiments. S. Lambrecht is a research assistant of the Fund for Scientific Research-Flanders. We acknowledge Dr. P. Verdonk and Prof. Dr. Almqvist for providing cartilage samples.

## References

- [1]Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., *et al.*, *Ann Intern Med* 2000, 133, 635-646.
- [2]Dieppe, P. A. and Lohmander, L. S., *Lancet* 2005, 365, 965-973.
- [3]Goldring, M. B., in: Klippel, J. H. (Ed.), *Primer on the rheumatic diseases*, Arthritis Foundation, Atlanta 2000, pp. 5-31.
- [4]Goldring, M. B., *Arthritis Rheum* 2000, 43, 1916-1926.
- [5]Vincourt, J. B., Lionneton, F., Kratassiouk, G., Guillemin, F., *et al.*, *Mol Cell Proteomics* 2006, 5, 1984-1995.
- [6]Garcia, B. A., Platt, M. D., Born, T. L., Shabanowitz, J., *et al.*, *Rapid Commun Mass Spectrom* 2006, 20, 2999-3006.
- [7]Ruiz-Romero, C., Lopez-Armada, M. J. and Blanco, F. J., *Proteomics* 2005, 5, 3048-3059.
- [8]Ruiz-Romero, C., Lopez-Armada, M. J. and Blanco, F. J., *Osteoarthritis Cartilage* 2006, 14, 507-518.
- [9]Molloy, M. P., Herbert, B. R., Walsh, B. J., Tyler, M. I., *et al.*, *Electrophoresis* 1998, 19, 837-844.
- [10]Gorg, A., Weiss, W. and Dunn, M. J., *Proteomics* 2004, 4, 3665-3685.
- [11]Verbruggen, G., Wang, J., Wang, L., Elewaut, D. and Veys, E. M., *Analysis of chondrocyte functional markers and pericellular matrix components by flow cytometry*, Humana Press, Totowa, New Jersey 2004, pp. 183-208.
- [12]Lambrecht, S., Verbruggen, G., Verdonk, P. C., Elewaut, D. and Deforce, D., *Osteoarthritis Cartilage* 2007.
- [13]Lambrecht, S., Verbruggen, G., Verdonk, P., Elewaut, D. and Deforce, D., *Osteoarthritis Cartilage*, doi: 10.1016/j.joca.2007.06.005.
- [14]Poole, C. A., Honda, T., Skinner, S. J., Schofield, J. R., *et al.*, *Connect Tissue Res* 1990, 24, 319-330.
- [15]Soder, S., Hambach, L., Lissner, R., Kirchner, T. and Aigner, T., *Osteoarthritis Cartilage* 2002, 10, 464-470.
- [16]Henrotin, Y., Kurz, B. and Aigner, T., *Osteoarthritis Cartilage* 2005, 13, 643-654.
- [17]Wang, M. X., Wei, A., Yuan, J., Trickett, A., *et al.*, *FEBS Lett* 2002, 531, 359-362.
- [18]Haslbeck, M., Franzmann, T., Weinfurtner, D. and Buchner, J., *Nat Struct Mol Biol* 2005, 12, 842-846.
- [19]Kappe, G., Franck, E., Verschuure, P., Boelens, W. C., *et al.*, *Cell Stress Chaperones* 2003, 8, 53-61.
- [20]Arrigo, A. P., Simon, S., Gibert, B., Kretz-Remy, C., *et al.*, *FEBS Lett* 2007.
- [21]Xu, L., Chen, S. and Bergan, R. C., *Oncogene* 2006, 25, 2987-2998.
- [22]Grimaud, E., Heymann, D. and Redini, F., *Cytokine Growth Factor Rev* 2002, 13, 241-257.

[23]Favet, N., Duverger, O., Loones, M. T., Poliard, A., *et al.*, *Cell Death Differ* 2001, 8, 603-613.



## **Chapter IV: Summary and future perspectives**



Osteoarthritis (OA) is the most common form of arthritis. In contrast to what would be expected by its name, this disease is generally considered to be a non-inflammatory joint disease. The major hallmark of OA is the progressive degeneration of the articular cartilage. Chondrocytes play a pivotal role in the normal metabolism of the cartilage matrix [1]. The failure to maintain the balance between synthesis and degradation of the extracellular matrix by the chondrocyte ultimately results in cartilage degeneration, distinctive for OA. As discussed in the introduction, a body of knowledge is available concerning cellular processes activated during the disease. In recent years, this knowledge was extended with data from the bio-molecular toolbox. However, the data from the proteome-level were unexisting or at least very limited.

The primary study was based on the hypothesis that the knowledge of differentially abundant proteins between chondrocytes isolated from ‘normal’ and OA-patients, may assist in unravelling novel disease processes activated in OA. So, the initial goal of this study was to identify differentially abundant proteins, using a 2-DE proteome approach. Such approach allows exploring the level of the effectors, namely the proteins. Moreover, it is an unbiased technique, thereby allowing the identification of novel targets. This study revealed the differential expression of several new players associated with processes known to be involved in the pathogenesis of OA, such as apoptosis [2], metabolic activation [3] and oxidative stress [4]. During this first study, we mainly focussed on two spots, both identified as vimentin. Based on spot patterns and mass spectrometry data, an enhanced N-terminal cleavage in OA-chondrocytes could be concluded. Given the involvement of the amino-terminal domain in intermediate filament assembly [5] and in membrane interactions [6], we investigated whether the observed cleavage influenced vimentin organisation. Indeed, a distorted vimentin organisation was visualized by immunofluorescence microscopy. The functional implications hereof may be of great interest. It is known that vimentin intermediate filaments may be involved in the visco-elastic properties of human articular chondrocytes [7]. In addition, chemical induced disassembly of the vimentin cytoskeleton, results in the disturbance of the normal chondrocyte homeostasis [8]. The importance of the cytoskeleton on mechanotransduction events was previously demonstrated [9]. Especially in the case of the chondrocyte, the cellular constituent of the weight bearing cartilage, these events could be very important. The primary cause of the observed vimentin cleavage remains elusive. Undoubtedly, the knowledge hereof will assist in the evaluation of the true biological value of our findings. Screening of the activity of intracellular proteases, which are known to target

vimentin, and inhibition of their activity may be a first step in future research. Furthermore, full characterization of the primary structure of the different vimentin isoforms may provide new insights and assist in further elucidation of the associated pathways.

In the second part of this study, we validated the differential expression of the small heat shock protein  $\alpha$ Bcrystallin and the functional implications thereof. This protein showed a gradual decrease in expression from healthy chondrocytes to chondrocytes isolated from visually damaged zones of OA-patients. Given the very limited knowledge concerning small heat shock proteins in chondrocytes, we further explored this very attractive target. One of the major hallmarks of OA chondrocytes is the involvement of pro-inflammatory cytokines in the activation of catabolic pathways [10]. Interestingly, the abundance of  $\alpha$ Bcrystallin showed to be IL-1 $\beta$  and TNF- $\alpha$  dependent, presenting this protein as a novel potential mediator of these key-cytokines in OA pathogenesis. Moreover these data implicate the potential involvement of a cytokine-driven mechanism in the suppression of  $\alpha$ Bcrystallin expression in OA chondrocytes. The observed decreased expression in OA chondrocytes was imitated by a siRNA induced suppression of the CRYAB gene expression. The application of high-efficiency knock-down technologies in chondrocyte research, revealed the association between a reduced  $\alpha$ Bcrystallin expression and the diminished expression of genes that are characteristic for the differentiated chondrocyte phenotype. Given the observed variable expression during chondrocyte dedifferentiation, this protein may be associated with the maintenance of the stable chondrocyte phenotype and its loss during the development of OA. The availability of an  $\alpha$ Bcrystallin knock-out model [11] opens up perspectives to investigate the in vivo role of this protein in cartilage biology. The application of OA models (e.g. collagenase-induced or surgically induced by anterior cruciate ligament transection [12]) on  $\alpha$ Bcrystallin knock-out and control mice might assist in further evaluation of this interesting gene. Moreover, future studies will have to reveal if modulating the CRYAB expression in chondrocytes will result in an increased synthesis of functional extracellular matrix components. Alternatively, modulating the expression of this gene in chondrocyte precursors or fibroblasts generated from expanded chondrocytes may result in the differentiation towards a more stable chondrocyte phenotype.

A final chapter describes an exploration of the proteome expressed by human articular chondrocytes. Sample prefractionation methods based on different protein and peptide



properties were combined and applied to different samples, resulting in the construction of a chondrocyte proteome database. Based on current and future knowledge of important pathways and differentially abundant protein species, this database could assist in the rational selection of candidate experimental targets. This was demonstrated by the selection of HSP27, a protein closely related to the previously described  $\alpha$ Bcrystallin. Future differential expression profiling experiments, whether targeted (e.g. Western blotting) or not (e.g. iTRAQ analysis), may aid in the evaluation of other targets (e.g. peroxiredoxins) provided by this proteome map. Alternatively, the provided proteome map may assist in the development of protein- or cDNA-arrays to evaluate expression levels of novel identified gene expression products. The ultimate goal of proteome profiling studies is to identify extremely low abundant proteins (e.g. cytokines or cytokine receptors in the particular case of chondrocytes). For cartilage samples, the amount of starting material is limited. Therefore, further technical improvements based on alternative fractionation methods and/or improvements in MS sensitivity are essential to assist in further digging in the chondrocyte proteome.

A major opportunity enabled by analyzing the protein level is the ability to discover post-translational modifications. In recent years it has become clear that PTMs are involved in the pathogenesis of several diseases (e.g. citrullination in RA). Therefore, specific profiling of PTMs such as phosphorylation (e.g. by selective enrichment of phosphoproteins) or protein cleavage (e.g. by N-terminal modification) may disclose novel disease-associated pathways and processes.

The initial 2-DE approach was the central feature of this study. The knowledge of the differential expression/abundance of a given protein or a given isoform on itself is interesting, but has limited biological value. Especially when the number of samples in the analyzed groups is limited, due to limited availability of patient samples, further validation is critical. Therefore, proteome analyses should be considered as discovery tools. The combination of different complementary proteome approaches, constitute a very important tool to kick-off the discovery process. Integration of these discovery tools with independent, multi-disciplinary approaches enabled us to present ‘novel’ proteins which may lead to new insights in the pathogenesis of OA. Hopefully, future research based on in vitro and in vivo data will elucidate the true biological role of these proteins.

## References

- [1]Goldring, M. B., *Best Pract Res Clin Rheumatol* 2006, 20, 1003-1025.
- [2]Sharif, M., Whitehouse, A., Sharman, P., Perry, M. and Adams, M., *Arthritis Rheum* 2004, 50, 507-515.
- [3]Hamerman, D., *N Engl J Med* 1989, 320, 1322-1330.
- [4]Henrotin, Y. E., Bruckner, P. and Pujol, J. P., *Osteoarthritis Cartilage* 2003, 11, 747-755.
- [5]Beuttenmuller, M., Chen, M., Janetzko, A., Kuhn, S. and Traub, P., *Exp Cell Res* 1994, 213, 128-142.
- [6]Georgatos, S. D., Weaver, D. C. and Marchesi, V. T., *J Cell Biol* 1985, 100, 1962-1967.
- [7]Trickey, W. R., Vail, T. P. and Guilak, F., *J Orthop Res* 2004, 22, 131-139.
- [8]Blain, E. J., Gilbert, S. J., Hayes, A. J. and Duance, V. C., *Matrix Biol* 2006.
- [9]Wang, N., Butler, J. P. and Ingber, D. E., *Science* 1993, 260, 1124-1127.
- [10]Goldring, S. R. and Goldring, M. B., *Clin Orthop Relat Res* 2004, S27-36.
- [11]Brady, J. P., Garland, D. L., Green, D. E., Tamm, E. R., *et al.*, *Invest Ophthalmol Vis Sci* 2001, 42, 2924-2934.
- [12]Stoop, R., Buma, P., van der Kraan, P. M., Hollander, A. P., *et al.*, *Osteoarthritis Cartilage* 2001, 9, 308-315.

## **Hoofdstuk IV: Samenvatting en toekomstperspectieven**



Artrose (OA) is de meest voorkomende gewrichtsaandoening in de westerse wereld. Deze aandoening wordt algemeen als een niet-inflammatoire pathologie beschouwd. OA is door een progressieve afbraak van het gewrichtskraakbeen gekenmerkt. De kraakbeencellen of chondrocyten, die autonoom instaan voor de synthese van de kraakbeenmatrix, spelen een sleutelrol in het metabolisme van het kraakbeen [1]. Algemeen wordt aangenomen dat een verstoring van het evenwicht tussen katabole en anabole processen leiden tot de afbraak van het kraakbeen, geassocieerd met OA. Er zijn reeds heel wat processen beschreven die in de pathogenese van OA betrokken zijn. De jongste jaren werd deze kennis door de snelle vooruitgang in de moleculaire biologie verder uitgebreid. Desalniettemin waren bij het begin van dit werk nagenoeg geen data beschikbaar op basis van proteoom-analyses in dit domein.

De werkhypothese, die de basis vormde voor deze studie, bestond erin dat de kennis van eiwitten die een verschillende abundantie vertonen in kraakbeencellen geïsoleerd uit normaal en artrotisch kraakbeen, nieuwe inzichten in de pathogenese van OA konden verschaffen. Het initiële doel van deze studie was dan ook om die eiwitten (of isovormen van eiwitten) te identificeren aan de hand van een differentiële proteoom-analyse op basis van 2-D gel elektroforese. Deze techniek biedt het voordeel dat het onderzoek wordt verricht op het niveau van de functionele componenten in een cel, namelijk de eiwitten. Bovendien hoeft men op voorhand niet te weten welke eiwitten onderzocht dienen te worden, wat dan weer toelaat om vernieuwende processen te ontdekken. Deze differentiële proteoom-analyse leidde tot de identificatie van nieuwe spelers in processen die algemeen gekend zijn in de pathogenese van artrose, zoals apoptose [2], metabole activatie [3] en oxidatieve stress [4]. Het verdere onderzoek in deze studie was hoofdzakelijk gericht op de validatie van twee differentiële spots, beide geïdentificeerd als vimentine. Een combinatie van massaspectrometrie- en Western blot-data toonde een verhoogde afbraak van de vimentine eiwitsequentie ter hoogte van het amino-terminaal gedeelte in OA-kraakbeencellen aan. Het is gekend dat dit amino-terminaal stuk van de eiwitsequentie betrokken is in de organisatie van het vimentine cytoskelet [5] en in de interactie van het eiwit met de celmembraan [6]. Immunofluorescentie-experimenten toonden inderdaad aan dat in OA-chondrocyten de vimentine-organisatie verstoord was. Deze bevinding kan mogelijks belangrijke functionele implicaties hebben op de cel. Men heeft namelijk beschreven dat het vimentine cytoskelet bijdraagt tot de visco-elastische eigenschappen van kraakbeencellen [7]. Bovendien werd aangetoond dat chemisch geïnduceerde afbraak van het vimentine cytoskelet resulteert in een verstoring van de metabole homeostase in kraakbeencellen [8]. Er werd verder ook aangetoond dat het

vimentine cytoskelet betrokken is in mechanotransductie-pathways [9]. Verder onderzoek zal moeten uitwijzen welke processen verantwoordelijk zijn voor de geobserveerde vimentine-afbraak tijdens de ontwikkeling van artrose. De kennis van deze processen zal ongetwijfeld verder bijdragen tot de evaluatie van het biologische belang van onze resultaten.

In het tweede gedeelte werd de geobserveerde differentiële expressie van  $\alpha$ Bcrystallin verder gevalideerd. Bovendien werd nagegaan wat de mogelijke functionele implicaties zijn van een gewijzigde expressie van dit gen. Vóór deze studie was weinig gekend over de functie en het gedrag van “small heat-shock proteins” in chondrocyten. Western blots bevestigden de lagere expressie van  $\alpha$ Bcrystallin in OA-stalen in vergelijking met chondrocyten geïsoleerd uit gezond kraakbeen. De betrokkenheid van pro-inflammatoire cytokines in de activatie van katabole processen is algemeen gekend in chondrocyten [10]. Tijdens onze studie observeerden we dan ook dat stimulatie van chondrocyten met pro-inflammatoire cytokines zoals IL-1 $\beta$  en TNF- $\alpha$  gepaard gaat met een lagere expressie van  $\alpha$ Bcrystallin. Dit kan erop wijzen dat dit eiwit een mogelijke mediator is van het effect van deze cytokines in chondrocyten. Bovendien wijst dit er ook op dat de lagere expressie van  $\alpha$ Bcrystallin in OA-chondrocyten wellicht het gevolg is van een cytokine-gedreven mechanisme. Om diepere inzichten te verwerven in de mogelijke functionele gevolgen van een gedaalde  $\alpha$ Bcrystallin-expressie werd de expressie van het CRYAB-gen onderdrukt door middel van “short-interfering RNA”. Deze gedaalde expressie bleek geassocieerd te zijn met een lagere expressie van genen die kenmerkend zijn voor een gedifferentieerde chondrocyt, namelijk BMP-2, collageen type II en aggrecan. Verder werd waargenomen dat dedifferentiatie van chondrocyten gepaard gaat met een initiële daling van de CRYAB-expressie, wat er op wijst dat dit gen bij het behoud van een stabiel fenotype van chondrocyten betrokken kan zijn.

De beschikbaarheid van een CRYAB “knock-out” model [11] opent nieuwe perspectieven om de rol van dit gen in vivo te bestuderen. De toepassing van OA-diermodellen (bv. collagenase-geïnduceerd of operatief geïnduceerd [12]) op deze knock-out muizen zal mogelijks een dieper inzicht geven in de rol van dit gen in de fysiologie van kraakbeencellen. Bovendien zullen toekomstige studies moeten aantonen of het mogelijk is om de chondrogene capaciteit van chondrocyten te beïnvloeden door de expressie van  $\alpha$ Bcrystallin te moduleren. Een andere mogelijkheid kan er in bestaan om de expressie van dit gen te moduleren in chondrocyt-precursoren of fibroblasten (gegenereerd uitgaande van chondrocyten tijdens expansieculturen) teneinde deze cellen te laten differentiëren naar chondrocyten.

In een laatste hoofdstuk werd het proteoom van chondrocyten verder onderzocht. Staal-fractionering gebeurde op basis van verschillende fysicochemische eigenschappen van de eiwitten en peptiden. Dit resulteerde uiteindelijk in een databank van eiwitten die in chondrocyten tot expressie komen. Het doel van deze databank bestaat erin om, op basis van gekende processen en gekende differentiële eiwitten, op een rationele manier nieuwe, mogelijke interessante, eiwitten te onderzoeken. Uit de databank bleek dat heat shock protein 27 (HSP27) tot expressie komt in chondrocyten. Dit eiwit is zowel structureel als functioneel zeer sterk verwant met  $\alpha$ Bcrystallin. De expressie van HSP27 werd dan ook verder onderzocht en vergeleken tussen normale en OA-chondrocyten. Verder differentieel onderzoek op basis van al dan niet gerichte kwantificatie (vb Western blotting of iTRAQ) zal ons in staat stellen om andere interessante eiwitten te evalueren. Bovendien kan deze databank een hulp zijn bij het ontwikkelen van eiwit- of cDNA-arrays om de expressieniveaus te evalueren van eiwitten die nog niet gekend waren in chondrocyten.

Het ultieme doel in dergelijke studies, die erop gericht zijn om het proteoom zo volledig mogelijk te beschrijven, bestaat erin om zeer laag abundante (vb cytokines of hun receptoren) eiwitten te identificeren. In het geval van kraakbeen is de hoeveelheid te isoleren eiwit beperkt. Verdere vooruitgang zowel op LC-MS vlak als op het vlak van staalvoorbereiding zal ons hopelijk in staat stellen om deze zeer laag abundante eiwitten te identificeren.

Eén van de belangrijkste voordelen van een proteoom-analyse bestaat in de mogelijkheid om post-translationele modificaties te identificeren. Het is algemeen gekend dat dergelijke modificaties betrokken zijn in de pathogenese van heel wat aandoeningen (vb. citrullinatie in RA). Het beschrijven van gemodificeerde isovormen, zoals fosforylatie of proteolytische afbraak van eiwitten, zal ongetwijfeld leiden tot de ontdekking van nieuwe ziekte-geassocieerde processen en mogelijks specifiekere biomerkers.

De initiële differentieële proteoom-analyse vormde de basis voor dit werk. De kennis van differentieel abundante eiwitten of eiwit-isovormen is interessant, maar heeft slechts een beperkte biologische waarde. Zeker in het geval van klinische stalen, die beperkt zijn in aantal, is verdere validatie absoluut noodzakelijk. Het is dan ook steeds duidelijker dat proteoom-analyses zeer waardevol zijn in het ontdekken van mogelijks interessante processen. De combinatie van complementaire proteoom-benaderingen kan deze informatie verder aanvullen. De integratie van de proteomics-discipline met andere technieken die complementaire informatie verschaffen hebben ons in staat gesteld om “vernieuwende”

eiwitten/genen als mogelijke mediators in de pathogenese van OA naar voor te schuiven. Verder onderzoek, gebaseerd op zowel in vitro als in vivo experimenten, zal ons hopelijk in staat stellen om de rol van deze eiwitten in OA verder te evalueren.



## Referenties

- [1]Goldring, M. B., Best Pract Res Clin Rheumatol 2006, 20, 1003-1025.
- [2]Sharif, M., Whitehouse, A., Sharman, P., Perry, M. and Adams, M., Arthritis Rheum 2004, 50, 507-515.
- [3]Hamerman, D., N Engl J Med 1989, 320, 1322-1330.
- [4]Henrotin, Y. E., Bruckner, P. and Pujol, J. P., Osteoarthritis Cartilage 2003, 11, 747-755.
- [5]Beuttenmuller, M., Chen, M., Janetzko, A., Kuhn, S. and Traub, P., Exp Cell Res 1994, 213, 128-142.
- [6]Georgatos, S. D., Weaver, D. C. and Marchesi, V. T., J Cell Biol 1985, 100, 1962-1967.
- [7]Trickey, W. R., Vail, T. P. and Guilak, F., J Orthop Res 2004, 22, 131-139.
- [8]Blain, E. J., Gilbert, S. J., Hayes, A. J. and Duance, V. C., Matrix Biol 2006.
- [9]Wang, N., Butler, J. P. and Ingber, D. E., Science 1993, 260, 1124-1127.
- [10]Goldring, S. R. and Goldring, M. B., Clin Orthop Relat Res 2004, S27-36.
- [11]Brady, J. P., Garland, D. L., Green, D. E., Tamm, E. R., et al., Invest Ophthalmol Vis Sci 2001, 42, 2924-2934.
- [12]Stoop, R., Buma, P., van der Kraan, P. M., Hollander, A. P., et al., Osteoarthritis Cartilage 2001, 9, 308-315.



## *Dank U!*

Deze doctoraats thesis is uiteraard tot stand gekomen dankzij de hulp van vele mensen. In de eerste plaats mijn (co-)promotoren Prof. Deforce en Prof. Elewaut: Jullie hebben me toegelaten om met de nodige zelfstandigheid dit project uit te werken en tegelijkertijd met een gezonde portie kritiek het werk bijgestuurd. Wat mij betreft mag deze samenwerking in elk geval nog een tijdje blijven duren. Daarnaast (en misschien nog veel belangrijker) hebben jullie er voor gezorgd dat er steeds voldoende financiële middelen om de toestellen en reagentia te voorzien die nodig waren. In dit opzicht bedank ik ook het FWO-Vlaanderen, die mij de voorbije jaren van de nodige financiële middelen heeft voorzien. Verder wens ik ook nog Prof. Verbruggen en Dr. Verdonk te bedanken: jullie hebben mij kennis laten maken met de kraakbeencellen in de praktijk. In het bijzonder bedank ik Dr. Verdonk en Dr. Almqvist voor de geregelde “levering” van de noodzakelijke stalen.

Een speciaal woordje van dank ook voor mijn collega's en mijn ex-collega's. In de eerste plaats de “(ex-)bewoners” van het 2<sup>e</sup> verdiep: Ben, Ivan, Aline, Kelly, Ann-Sophie, Maarten, Jip, Mado, Katleen en Cynthia. De sfeer, maar ook de gewone ‘goeiemorgen’, de laatste roddelkes, het koffietransport naar boven, de discussies (variërende van de buffer-keuze voor de LC tot de selectie van het beste bier) en zo veel meer heeft me de voorbije jaren zeer veel plezier gedaan. De discussies over de organisatie/opkuis (nu ja, veel ruimte (letterlijk, dan) om te organiseren was er misschien niet altijd...) liepen soms hoog op, maar telkens kon de vrijdag-avond-duvel (gekoeld à la façon “2<sup>e</sup> verdiep”) de rust doen terugkeren. In elk geval: mochten jullie ooit vinden dat jullie bureau er belachelijk leeg uit ziet, ik zal met plezier eens langskomen... Filip, jij hebt wel nooit het 2<sup>e</sup> verdiep bevolkt, maar voor jou geldt zeker hetzelfde als hierboven.

“Sofie van de massa”: super-veel bedankt voor de hulp de voorbije jaren. Jouw werk apprecieer ik ten zeerste. De finesse en vooral het geduld waarmee je een naaldje kan positioneren, de lekken kan “dichten”,... is ongeëvenaard binnen het lab. We hebben veel moeten uittesten en veel gesukkeld, maar jouw hulp heeft mij veel tijd bespaard.

Uiteraard ook de mensen van “den DNA”: jullie hulp zat in kleine dingen, maar daarom niet minder belangrijk. Enne niet te vergeten, Astrid en Nadine: de bestellingen, faxen, administratie en zo veel meer, bedankt hiervoor.

Daarnaast zijn er nog een reeks mensen die af en toe of tijdelijk in ons labo langskwamen, zoals de thesisstudenten, de MRB-people,... (wat dan op zich weer een gelegenheid kon zijn voor één of meerdere gezamenlijke etentjes, MRB-BBQ, feestjes...), ook jullie bedankt voor de voorbije jaren.

Zonder ontspanning, ook geen inspanning, dus naast de bovenvermelde mensen zijn er nog heel wat familie en vrienden die een indirecte bijdrage hebben geleverd aan het welslagen van dit werk, dus ook jullie bedankt. In het bijzonder nog een zeer speciaal woordje van dank voor “ma en pa”. Zonder jullie was ik hier nooit geraakt, dus een zeer welgemeende merci! Bieke, bedankt voor de taal-technische aanpassingen aan de Nederlandstalige tekst en voor zoveel meer... Het is moeilijk om in een dankwoord niet in clichés te spreken, en waarom moeilijk doen als gemakkelijk evenveel zegt: Last but not least, Wennie, bedankt voor zoveel dingen de afgelopen jaren!

